Association of *Salmonella* with *Arachis hypogaea* (peanut plants and seedpods)

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ABSTRACT

Salmonella is the leading cause of bacterial foodborne illness in the United States and its presence in food results in the recall of many products every year. An increasing concern in the food industry is the contamination of peanuts with *Salmonella*. The goals of this research were to determine if *S.* Typhimurium was capable of internalizing in peanut seedpods and plants and identify factors involved in this uptake such as moisture, seedpod status, soil type and the influence of the peanut symbiont, *Bradyrhizobium*.

Intact dry Virginia (DV) seedpods were exposed to *S.* Typhimurium suspensions and inoculated soil under different conditions. *S.* Typhimurium suspensions containing 7 Log CFU/mL were examined for different times, and temperatures. DV and green Virginia (GV) seedpods were also exposed to potting media and Hubbard series soil inoculated with 6.5 Log CFU/g. The initial moisture content of each soil was adjusted and the results were compared. Internalization was measured by swabbing and rinsing the interior surface of the seedpods following exposure. *S.* Typhimurium was quantified using differential tryptic soy agar (dTSA).

Internalization of *S.* Typhimurium from soil into peanut plants was also examined. Soil was inoculated with 6 Log CFU/g. Sterile seeds were sowed and grown for 35 days after inoculation. Following surface sterilization, whole plants were divided into root, stem, and leaf samples. Each plant section was homogenized and quantified using dTSA. For experiments involving *Bradyrhizobium*, seeds were dip-inoculated in a suspension containing 8 Log CFU/mL of *B.* NC92 and then tested as described above.

Intact DV seedpods were susceptible to *S.* Typhimurium internalization over all of the temperatures and times tested when immersed in cell suspensions. After exposing seedpods to the suspension for 22 h, *S.* Typhimurium was recovered internally at a level of 6.4 Log CFU/pod. Internalization also occurred rapidly in as little as 0.5 h. Significant differences were observed between the recovery of *S.* Typhimurium from the internal surface of DV and GV seedpods when exposed to soil. Overall differences were also identified in the ability of *S.* Typhimurium to infiltrate seedpods when delivered through potting media and Hubbard series soil at specific soil moisture contents.

S. Typhimurium was capable of internalizing in peanut plant tissues and remained present at all testing times present. *S.* Typhimurium was recovered from stem samples (3.5 Log CFU/g) at greater levels than was observed for root (2.6 Log CFU/g) and leaf (1.7 Log CFU/g) samples. Overall results for stem, root, and leaf samples were recovered at lower levels when *B.* NC92 was inoculated on seeds before sowing. However, this difference was not significant for any time point, or plant section.

Overall, this study's results suggested the importance of water for *S.* Typhimurium to internalize within peanut seedpods. Moreover, the initial soil moisture content in relation to the water-holding capacity also impacted the ability of *S.* Typhimurium to internalize in seedpods. This work also observed that *Salmonella* was capable of internalization in peanut plants through inoculated soil. Moreover, the results indicated that the nodulating symbiont, *B.* NC92, did not significantly influence the internalization of *S.* Typhimurium when seeds are sowed in inoculated soil. This work provides some of the first evidence that peanut seedpods and plants are susceptible to

Salmonella internalization, which may represent a potential route of entry of *Salmonella* into a processing facility from the field.

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INTRODUCTION

Salmonellosis, a disease caused by the enteric bacterium *Salmonella* is responsible for as many as 1.03 million infections in the United States (US) annually, which results in an estimated 378 deaths (1). The large number of cases, along with the fact that immunocompromised individuals such as children and the elderly are more susceptible to the disease, makes the impact of salmonellosis on human health great. Indeed, current estimates of economic losses due to *Salmonella* illness range from 3.7 to 5.5 billion dollars in the US annually (2, 3). As such, a fundamental understanding of the relationship between the pathogen, its environment, and its ability to enter the food supply is crucial to outbreak prevention.

While peanuts and peanut-containing products have been associated with several significant *Salmonella* outbreaks, *Salmonella* contamination with peanuts has only recently been reported. The first record of a *Salmonella*-related outbreak in peanuts occurred in 1994 when 71 were stricken with salmonellosis related to the consumption of peanut flavored snacks distributed in Israel, the United Kingdom (UK), and the US (4). Since then, 7 others have been reported throughout the world, one of which resulted in over 700 illnesses and contributed to 9 deaths (5, 6). The impact of an outbreak is further increased as the consumption of peanut butter in the US was reported to be 6.7 pounds per person in 2012 (7). This equates to over 2 billion pounds being consumed annually. It is also estimated that US production of peanuts will top 6 billion pounds in 2015 (8). With such large quantities being produced and consumed, if a significant contamination issue occurs the impact on human health could be greater than previously observed.

Due to the pathogen's ubiquitous nature, *Salmonella* contamination with food and water is common. For many years, it was assumed that low water activity foods such as peanut butter were assumed to be free of human pathogens. It was thought that *Salmonella* and other pathogens could not survive under low water activity conditions. However, the large number of outbreaks that have occurred in recent years has demonstrated that *Salmonella* is capable of not only surviving at low water activities but also surviving for extended periods of time (9). The behavior of *Salmonella* in the environment and in it survival in different food matrices has been studied to some extent, but the interaction between peanut plants, seeds, and *Salmonella* has not been fully examined to understand the potential of *Salmonella* contamination in a processing facility through this route. The notion that *Salmonella* can come to be internalized in plants is a newly observed phenomenon, dating back to 2002 (10). This observation has been studied in detail for plants such as lettuce and tomatoes, but this relationship in peanut plants remains incomplete.

The goal of this research project was to assess the occurrence of *Salmonella* internalization into peanut plants and seedpods. The first specific objective was the characterization of *Salmonella* internalization through intact peanut seedpods in both inoculated suspensions and soil. This part of the project also included the assessment of factors that influenced this trait. The second objective was to examine the ability of *Salmonella* to internalize in growing peanut plants. After establishing the ability of *Salmonella* to internalize peanut plants, the impact of the nitrogen-fixing symbiont, *Bradyrhizobium*, was also assessed.

CHAPTER 1: LITERATURE REVIEW

1.1 *Salmonella* – organism characteristics

1.1.1 General characteristics

Bacteria from the genus *Salmonella* are facultatively anaerobic Gram-negative rods. Like other organisms from the *Enterobacteriaceae* family, *Salmonella* is a chemoorganotroph and is limited to the utilization of organic compounds as energy sources (11). Members typically contain peritrichous flagella and are motile, but some non-flagellated variants do occur such as *S.* Pullorum and *S.* Gallinarum (11, 12). As an enteric pathogen, salmonellae grow optimally at 37°C, but are also able to grow and survive at a wide range of temperatures. *Salmonella* is able to grow at temperatures as low as 2°C to and it has been reported to duplicate at 54°C (11). This wide temperature range is only observed if cells are pre-conditioned for growth at such temperatures. *Salmonella* can use a variety of substrates for growth, but lacks the ability to ferment lactose unless the necessary genes are present in a plasmid (11, 12). All of these characteristics make *Salmonella* a unique and diverse genus of bacteria.

1.1.2 Nomenclature

Taxonomically, the *Salmonella* genus is quite diverse, consisting of over 2,500 currently recognized serovars residing in only two species (12). This number will no doubt continue to increase due the use of whole genome sequencing, which allows for further differentiation among strains. The two existing taxonomical species are *Salmonella enterica* and *S. bongori*. *S. enterica* includes 6 subspecies from which *S. enterica* subsp. *enterica* (I) is the most frequent cause of foodborne outbreaks as 99% of human isolates belong to this subspecies (12, 13). *S. enterica* subsp. *enterica* (I) is also very diverse, containing more than 1,400 serovars. A wide variety of these serotypes have

been implicated in foodborne disease, but approximately 70% of *Salmonella* isolates from humans are from only 20 different serotypes (12, 13). Serovars Typhimurium, Bredeney, Mbdanka, and Tennessee have all been implicated in outbreaks related to peanut products and are of particular importance to this project.

1.1.3 Important physiological characteristics

A few significant physiological traits help distinguish *Salmonella* from other microorganisms. Generally, salmonellae possess the enzyme thiosulfate reductase, which reduces thiosulfate to produce hydrogen sulfide (14, 15). This trait is widely used as a method to differentiate *Salmonella* from other enteric organisms. Salmonellae also possess the ability to decarboxylate lysine, producing cadaverine which is highly basic (11). Other key features of *Salmonella* include its ability to use citrate as its sole carbon source and also that salmonellae are catalase positive, oxidase negative, and deficient in the ability to hydrolyze urea (11). All of these physiological traits are used in combination to distinguish *Salmonella* from other microorganisms. Even with the advent of molecular detection methods, biochemical traits are still widely used owing to the fact that genetic methods are still regarded as presumptive and require confirmation using biochemical screening (16).

1.1.4 Analysis and detection of *Salmonella*

As mentioned previously, *Salmonella* detection based on traditional cultural and biochemical methods continue to be an important means of *Salmonella* identification in the food industry. Typical salmonellae are isolated first by resuscitating and growing cells in pre- and secondary-enrichments. Isolation is then accomplished by streaking enriched samples onto complex media that selects for and differentiates *Salmonella* (17,

18). A key trait of *Salmonella* that has been exploited by scientists is its ability to reduce thiosulfate to hydrogen sulfide which then quickly reacts with ferric iron present in the medium that converts the colorless gas to iron sulfide (17). Iron sulfide is a black precipitate and is characteristic of *Salmonella* colonies on complex media which allows for the differentiation of *Salmonella* compared to other enteric pathogens such as *Escherichia coli*.

Traditional microbiological isolation techniques, such as those described above, are often constrained by time making the need to develop easier and faster methods an important task for the food industry. As such, many rapid detection methods offering a high degree of specificity and fast turn-a-round times have been developed. Due to the emergence and popularity of new rapid detection methods, validation programs have also grown to be a vastly important aspect of method validation. The most widely used method validation scheme was developed in collaboration between the International Organization for Standardization (ISO) and AOAC. These tests rely on different techniques, such as immunodiffusion, immunofluorescence, or the polymerase chain reaction.

1.2 ***Salmonella*** **in the environment**

1.2.1 Prevalence and spread of *Salmonella* in the environment

As an enteric pathogen that continually goes through periods of environmental exposure, the fit and fitness of *Salmonella* in specific environments holds a great deal of interest to the scientific community. Some of the earliest work on the topic established a 99% reduction of *Salmonella* inoculated poultry excreta within 3 days at 30°C, but noted

persistence for as long as 148 days at 11°C (19). Similarly, when parsley was spray inoculated with *Salmonella* Typhimurium, the pathogen was detectable in the phyllosphere, rhizosphere, and soil for at least 28 days, indicating that the organism is able to survive for extended periods in a plant environment (20). In 2013, it was found that *S.* Newport had a particular fitness in a soil environment, as this serovar was recovered from 100% of tomato rhizosphere samples at 23 days post inoculation (21). Contrast this with *S.* Typhimurium, which was undetectable in soil at 23 days post inoculation in the same study reference previously (21). This finding was compared with Bernstein et al. that produced similar results as *S.* Newport was able to persist in potting media from 4.7 to 10 weeks after inoculation (22). Although there are numerous factors that influence the presence and survival of *Salmonella* in a given environment, the pathogen displays the ability to survive and persist in the environment for extended periods of time.

A common thread observed in the studies mentioned previously is that *Salmonella* can survive for an extended period of time when exposed to different environments. A study of four different watersheds in North Carolina reported that *Salmonella* was isolated from watershed samples at a rate of 54.7% (23). That work showed no significant difference among the four watershed types taken (swine production areas, crop agricultural areas, forestry areas, and residential/industrial areas) indicating that the prevalence of *Salmonella* is not influenced by the proximity of the watershed to other factors. Likewise, a study looking at the seasonality in watersheds demonstrated that the *Salmonella* populations reached their maximum during the summer months compared

with other seasons (24). Similarly, samples positive for *Salmonella* were isolated at higher rates at the highest water temperatures.

These reports isolated *Salmonella* at high rates from watershed environments in the Southeast United States. To contrast this, Micallef et al. isolated *Salmonella* in 12 of 1091 (1.1%) samples taken from tomato farms in the mid-Atlantic region of United States (25). In a more extensive study of 2,496 samples from 18 farms across 5 states, *Salmonella* serovars were recovered from 4.7% of all samples (26). The study also noted that *Salmonella* was more frequently found in swine farms than dairy, poultry, or cattle farms. The evidence provided demonstrates the high degree of variability in detecting the pathogen in the environment, which in part may be affected by both protozoan predation and temperature (27).

1.2.2 Natural reservoirs of *Salmonella*

Salmonella is a common enteric pathogen, and a natural inhabitant of the gastro-intestinal tract of animals including amphibians, reptiles, birds, and mammals. Although most serovars are pathogenic to humans, of the majority serovars do not actually cause symptoms in the animal host and are simply transient. Because of the high consumption of poultry throughout the world, the most important reservoirs of *Salmonella* have been domesticated chicken (*Gallus gallus domesticus*) and turkey (*Meleagris gallopavo*). Cox et al. noted that the prevalence of *Salmonella* in breeder hatcheries is quite variable, with a positive rate of 1.3% in one hatchery to as high as 36% (28). Regarding the general incidence of *Salmonella* in turkeys, a study that tested 25 flocks indicated that infected birds ranged from 0% to as high as 72% (29). Thus, it has been concluded that the

colonization of *Salmonella* in these animals is largely due to exposure to other infected individuals.

Other important reservoirs mentioned above are amphibians and reptiles. Amphibians are not typically consumed as food, but various species are known to harbor *Salmonella*. Turtles are of particular importance, due in large part to its role as pets for children. A study of two species of turtles native to Spain found that 11% of these turtles harbored *Salmonella*; in particular *S.* Thompson and *S.* Typhimurium were the most prevalent serovars (30). Due to the relatively high prevalence rate, these animals can play an important role in perpetuating the existence of *Salmonella* in specific environments.

Extensive research has been aimed to understand the factors that contribute to *Salmonella* expulsion from the host into the environment. Various studies have investigated how feedlots affect *Salmonella* shedding patterns over time in beef cattle (31-34). The results of some of these studies were contradictory with some suggesting that *Salmonella* prevalence increased with time at feedlots, while others suggest that *Salmonella* prevalence stays constant or even decreases over time. In one study examining the affect of feeding, transportation and holding on *Salmonella* prevalence in market-age turkeys, no significant difference was observed among the groups tested (35), which suggested that *Salmonella* shedding was affected by other factors. A model developed to analyze factors affecting shedding in pigs indicated that shedding is dose and serovar dependent (36). Overall, the notion that *Salmonella* shedding is intermittent and some individuals play a role as carriers infecting an entire group of animals is now generally accepted (32).

1.2.3 *Salmonella* in food processing plants

Given that there have been 493 recalls of foods due to *Salmonella* contamination since 2010 (37), the presence of *Salmonella* in food processing facilities is well established. The factors that influence the ability of *Salmonella* to survive the processing environment are an active area of interest to the scientific community. Many of the outbreaks associated with *Salmonella* are often related to the pathogen's ability to survive in harsh environments. Thermal, desiccation, and acid resistance are three of the most common and significant adaptations that *Salmonella* can use to persist in a processing facility. The attributes of *Salmonella* will continue to make the control of this pathogen a food industry concern.

Studying the behavior of *Salmonella* in different environments, scientists have noted that prior exposure to certain conditions, will often offer *Salmonella* cross-protection against other conditions. This means that exposure to one environmental stress results in the organism developing resistance to another environmental stress (11). The idea of cross-protection is critical when understanding *Salmonella* in the environment. Rarely will any organism be exposed to one condition. As an example, *S.* Typhimurium cells pre-exposed to the mildly acidic conditions (pH of 5.8) were found to have an increased tolerance to osmotic and thermal stress, as well as polymyxin B, which acts on the outer membrane of the bacterium (38). Concerning desiccation, it has been reported that temperature affects *Salmonella* survival (39). *Salmonella* cells exposed to 5°C survived for 22 to 24 months, whereas at 25°C, *Salmonella* only survived for 35 to 70 days. Moreover, cells exposed to conditions of desiccation acquire an enhanced ability to withstand thermal treatment. When *Salmonella* was mixed with chicken litter, the

bacterial population that had been exposed to mild desiccation conditions, a_w of 0.87, took an average of 2.5 hours longer to decrease 5 logs than non-exposed cells (40). The mechanism involved in this adaptation, however, remains elusive. The unique traits that *Salmonella* possesses makes it well adapted to withstand multiple environmental stresses.

1.3 *Salmonella* interaction with plants

Certain vectors of *Salmonella* such as animals, contaminated irrigation water, or even aerosols may allow the bacterium to gain entry onto plant surfaces. This is where the interaction of *Salmonella* with plants begins. The plant contains drastically different environments, of which *Salmonella* has to cope with a number of different stresses in order to survive. The plant phyllosphere is the portion of the plant above ground and provides its own unique set of stresses that impact *Salmonella* growth and survival.

Salmonella has been reported to be capable of growth on plant surfaces in wet and warm conditions (41, 42). This trait was displayed only under ideal conditions, as cooler, dry conditions did not normally result in the growth of the pathogen and can even lead to a decrease in the population (43). Thus, it is more than likely that *Salmonella* can grow intermittently under warm, wet seasonal conditions, but then remain static or even decrease in number under dry conditions (42, 43). Competition for nutrients appeared to be a factor preventing the growth of *Salmonella* in the leaf environment (42), although the impact of nutrient limitation is yet to be fully examined. *Salmonella* was also able to aggregate towards the veins of leaf tissue (43). Researchers speculated to be due to the increased amount of water available to cells in that region and perhaps the profusion of carbohydrates as well (42, 44).

The ability of *Salmonella* to grow in this environment seems to be highly variable and often circumstantial, but if the organism is able to survive in the plant environment an outbreak can still occur. Several studies have demonstrated that *Salmonella* has the ability to persist in the plant phyllosphere for extended periods (45-47). Islam et al. reported that *S.* Typhimurium was detectable on lettuce for 63 days after inoculation on lettuce and for 231 days after inoculation on parsley (45). Considering that the bacterium can remain viable after such long periods, the factors that allow the pathogen to survive in this environment have also been studied.

The attachment of *Salmonella* to plant surfaces is an important area of study, as it has implications in the development of effective mitigation strategies for pathogen control. Mutations in the *Salmonella* genome involving *agfB* (48) and *ycfR*, *sirA*, and *yigG* (49) have been reported to be important in the attachment of the pathogen to the surface of spinach plants. Moreover, YcfR was reported to play an important role in the resistance of the bacterium to hypochlorous acid (49). This important genetic trait aids in the survival of the bacterium in high stress environments such as those encountered in a produce processing facility.

Another critical aspect of the interaction between plants and *Salmonella* is the ability of the bacterium to associate with the plant rhizosphere, or the area of the plant that is below ground. This environment is thought to be an important route of entry of *Salmonella* to internalize within plant tissue. *Salmonella* was able to grow extensively to 9 Log CFU/g on *Arabidopsis thaliana* roots after being inoculated at 6 days post-germination in a sterile environment (50). Five days after the inoculation of *S.* Typhimurium at a level of 3 Log CUF/plant in a plant medium containing *Medicago*

sativa seedlings, the population within the rhizosphere reached 6.5 Log CFU/plant (51). In controlled environments, as in the cases mentioned above, *Salmonella* was capable of growing within the plant rhizosphere.

In a non-sterile rhizosphere, however, the amount of recoverable *Salmonella* declined throughout the four week testing period in growing parsley when inoculated in non-sterile potting media (20). Similar to its survival in the phyllosphere, *Salmonella* was persistent in the rhizosphere for the duration of the study (20). Whether or not there is an active mechanism which *Salmonella* uses to orient itself towards the rhizosphere is still being examined. Klerks et al. demonstrated that *Salmonella* exhibited active chemotaxis towards root exudates of lettuce in microcapillaries (52). In a plant-soil model, similar results have yet to be observed, but in the experiments performed by Kisluk et al. there was no difference in the levels of *Salmonella* between the rhizosphere and exterior soil (20). This finding indicates that chemotaxis may not be important in the ability of *Salmonella* to colonize the rhizosphere and likely depends on the environment.

The knowledge of endophytic microorganisms was first discovered in the 1870's when Louis Pasteur and others observed bacteria in plant tissue without any outward symptoms, but it wasn't until the 1940's that the study of plant endophytes began to expand (53). After several decades of study involving microorganisms relating specifically to plant and soil, the relationship between plants and human pathogens has been recently examined. In 2003, a study by Coolley et al. indicated that *Salmonella* was capable of moving throughout the vascular tissue of *Arabidopsis thaliana* when inoculated through the root system (50). It wasn't long after that Warriner et al. provided more evidence that *Salmonella* also has this ability in mung bean sprouts (12). They also

reported that internalization prevented the removal of the pathogen through the use of washing (54). Thus, it has been observed that under the right conditions, *Salmonella* is capable of becoming a plant endophyte.

Extensive research has been conducted on the cultivation of tomato plants and how *Salmonella* interacts with this environment. Guo et al. suggested that salmonellae was capable of an endophytic relationship when tomato plants were grown hydroponically in an inoculated solution (10). Plants inoculated with 7.0 Log CFU/ml every seven days were shown to contain internalized cells at a rate of 27% (55). After establishing that *Salmonella* can become an plant endophyte, it was later observed that the serovar present was an important variable that affected plant internalization (21). In a study published in 2013, *Salmonella* Newport was the only serovar found inside and on the surface of a tomato fruit when plants were transplanted into *Salmonella* containing soil (21).

Peanut plants were also examined for *Salmonella* internalization. It was found that internalized *Salmonella* were localized throughout the vascular tissue of the plant when seeds were inoculated with 10^6 CFU/seed (56). All of these results indicated that *Salmonella* was able to internalize in not only sprouts, but also larger crops as well. The mechanism by which *Salmonella* is able to internalize plants via the root system remains elusive at this time. There are some bacteria capable of using type three secretion systems (T3SS) to introduce effectors to both plants and animals, but this ability has only been shown to be used by *Salmonella* in animals (57). At this time, there is no evidence to suggest for or against the use of T3SS as a factor influencing the ability of *Salmonella* to

internalize in plants. Other properties of the mechanism by which *Salmonella* enters plant tissue have not been elucidated.

In controlled conditions, *Salmonella* was able to internalize within plant tissue from the root system (10, 21, 55). There are other routes, however, for *Salmonella* to gain entry into plant tissue. One important access point for cells appears to be through the stomata of plant leaves. *Salmonella* was able to gain entry through stomata, in a process that involves bacterial chemotaxis (58). Motility mutants, devoid of the *fliGHI* gene, had a reduced ability to internalize in lettuce leaves. Similarly, mutants defective in the *cheY* gene for bacterial chemotaxis were significantly inhibited in their ability to internalize a lettuce leaf (58). Although the closing of stomata is a part of the plants immune response to minimize invading bacteria, plant pathogens are known to possess specific virulence factors which can overcome stomatal closing (59, 60). Whether or not *Salmonella* follows a specific mechanism to bypass this innate immunity is still unknown.

1.4 *Salmonella* infection

1.4.1 Mechanism of infection

Salmonella infections typically occur after oral ingestion, as is this case during the regular consumption of food and water. Ingestion of *Salmonella* can also occur as a result of exposure to contaminated surfaces and pets that carry *Salmonella*, such as reptiles or amphibians (30). After ingestion, *Salmonella* is exposed to the low pH of the stomach. *Salmonella* possesses an acid tolerance response mechanism that promotes its passage from the low pH of the stomach to the small intestine (61). Upon entering the lower intestine, the terminal ileum has been shown to be the primary attachment site for

Salmonella (62). Furthermore, it was also established that *Salmonella* shows a high degree of specificity to lymphatic masses known as Peyer's Patches.

Although it has been shown that *Salmonella* can degrade and cause membrane rearrangements of microvilli which facilitate absorption by non-phagocytic enterocytes for epithelial translocation (63), the phagocytic microfold cells or M-cells are the preferred site for *Salmonella* to gain entrance to the *lamina propria* (64). After exiting the lumen of the intestine, *Salmonella* can migrate to the mesenteric lymph node (MLN) extracellularly or more likely due to transport via dendritic cells (65). Entry to the MLN will allow its dissemination to other tissues of the reticuloendothelial system such as the liver or spleen (65). However, infections of healthy adults caused by non-typhoidal strains are generally limited to the intestine and rarely result in infection of other tissues (66).

To cause infection, *Salmonella* must evade the innate immune system response of the host. Within the intestinal lumen the bacterium first encounters phagocytic cells known as macrophages (67, 68). To evade subsequent macrophage digestion and invade epithelial cells, *Salmonella* has developed a number of defense responses that allow the bacterium to grow and proliferate within the host environment. Of particular importance are the genes encoded on *Salmonella* pathogenicity islands I and II (SPI and SPII). Both SPI and SPII contain the genes encoding type III secretion systems (T3SS). A T3SS is a specialized apparatus that allows the bacterium to transfer virulence proteins into host cells (69). The transferred proteins can then alter host cell function to aid in the survival and proliferation of the bacterium. The evolution of the T3SS overall is one of considerable debate owing in large part to its similarity to the bacterial flagella (70-72). A

25 kb portion of SPII encodes for a T3SS that is important for virulence (73). This portion of SPII is only found in *Salmonella enterica* and is viewed as a critical evolutionary step in the pathogenicity of *Salmonella*, as *S. bongori* is seldom related to human disease and is a phylogenetically older species (73).

1.4.2 Human disease characteristics

Salmonella is responsible for several diseases including typhoid fever, enterocolitis, and also systemic infection. Typhoid fever is caused by *Salmonella* serovars Typhi and Paratyphi and is transmitted human to human via the fecal-oral route (11, 12). The disease has an incubation period of 7 to 28 days and induces symptoms such as abdominal pain, diarrhea, fever, headache and prostration (74). If left untreated, the mortality rate for typhoid fever can be up to 10% (75). Supportive therapy is generally used for treatment such as fluid and electrolyte replacement, although advanced cases my require antibiotic treatment (11). An increase in antibiotic resistant typhoid strains has made treatment with antibiotics increasingly difficult. Other avenues to combat this resistance will continue to be explored, such as the use of phages capable of suppressing infection (11, 76).

More relevant to the food industry and developed countries is the enterocolitic disease termed salmonellosis. The disease is generally self-limiting, lasting anywhere between 4 to 7 days and symptoms developing after 12 to 72 hours from the initial ingestion of the bacterium (75, 77). Supportive therapy is used to treat the disease, but antibiotic treatment is typically avoided because it can result in prolonged excretion of *Salmonella*. Although salmonellosis is generally self-limiting, systemic disease can occur, but the frequency of systemic infections has not been established.

1.4.3 Impact of disease

Non-typhoidal *Salmonella* is estimated to cause between 640,000 and 1,700,000 cases in the United States annually (1). The CDC has calculated that 94% of all *Salmonella* cases are foodborne. On a global scale, Majowicz et al. has estimated that non-typhoidal *Salmonella* infects between 61,800,000 to 131,600,000 with an estimated 38,000 to 300,000 deaths per year (78). With over 1,000,000 estimated infections in the US per year, the human and financial impact that *Salmonella* causes is great. Compiled in Table 1 is a list of the estimated monetary loss per case of selected foodborne pathogens. Minor et al. estimated that the average monetary loss from non-typhoidal *Salmonella* is $5,337 per case (2). Given that *Salmonella* is estimated to infect just over 1,000,000 individuals each year, the annual monetary loss due to salmonellosis is approximately $5.5 billion (2). In 2014, a subdivision of the United States Department of Agriculture (USDA), the Economic Research Service (ERS), published cost estimates of foodborne illnesses for select pathogens. The ERS estimate puts the total cost of non-typhoidal *Salmonella* illnesses at $3.7 billion annually (3). The ERS estimate takes into account lost wages, outpatient and inpatient expenditures, and estimates of the general public's "willingness to pay." Willingness to pay is a metric designed to estimate how much individuals will pay to minimize pathogens in their food supply (79, 80). Although the two sources differ, the scale of monetary losses due to *Salmonella* infections is surely in the millions. Losses to individuals as a result of foodborne disease have been widely studied (2, 3, 79-81). There is very little information concerning the cost of foodborne outbreaks and recalls to food companies. This is likely due in part to the variability of

components that are hard to quantify such as brand image, cost of lawsuits, and any facility redesign that may occur as a result.

Table 1: Estimated cost and economic burden of foodborne illnesses on a per case basis. Adapted from Minor et al. (2)

	Monetary Loss (in dollars)		
		95% Confidence Interval	
Pathogen	Mean	Lower Tail	Upper Tail
Bacteria			
Bacillus cereus	208	201	215
Campylobacter spp.	3,488	2,662	4,903
Clostridium botulinum	1,514,289	1,503,300	1,525,594
Clostridium perfringens	210	204	218
Escherichia coli O157:H7	10,274	7,814	12,668
Listeria monocytogenes	1,456,676	1,445,112	1,468,384
Salmonella spp. (nontyphoidal)	5,337	4,868	5,915
Salmonella enterica Typhi	5,487	4,915	6,060
Shigella spp.	2,800	2,145	3,506
Staphylococcus aureus	376	294	459
Vibrio vulnificus	3,671,276	3,661,931	3,680,555
Vibrio parahaemolyticus	1,904	1,493	2,313
Yersinia enterocolitica	4,566	3,400	5,753
Parasites			
Cyclospora cayetanensis	3,252	1,181	5,298
Toxoplasma gondii	41,652	38,751	44,492
Trichinella spp.	12,135	5,255	19,034
Viruses			
Hepatitis A virus	42,780	41,430	44,105
Norovirus	363	281	445

1.5 ***Arachis hypogaea* Linneaus (L.) – plant characteristics**

1.5.1 General characteristics

Arachis hypogaea L., or peanut, is not a true nut as the name suggests, but rather a legume residing in the family of plants known as *Fabaceae*. The *Arachis* genus contains 15 species, but *A. hypogaea* is the only cultivated species (82). *A. hypogaea* is further divided into 2 subspecies: *hypogaea* (runner) and *fastigiata* (bunch). The distinction between these two subspecies resides in the unique characteristic of the *fastigiata* subspecies to produce flowers on the main stem, whereas the *hypogaea* subspecies does not (83). The *fastigiata* subspecies also requires less water and produces a smaller seed than the *hypogaea* subspecies. There are four major classes of peanut varieties currently cultivated in the US, the Virginia and runner types, belonging to the *hypogaea* subspecies, and the Spanish and Valencia types, belonging to the *fastigiata* subspecies (83). Bunch type peanuts grow to as high as 60 cm, while runner types grow shorter to a maximum of only 45 cm (82).

Arachis hypogaea L. produces flowers that are small and pea-shaped and develop roughly 6 weeks after sowing. Flowers are self-pollinating, which generally occurs within 24 hours of blooming. Cross-pollination does occur at low rates (1 to 6%) due to the pollination of bees or atypical flower development (84, 85). Legumes are a family of plants that produce fruit in pods and are often capable of obtaining an independent nitrogen source through a symbiosis with rhizobial species of bacteria. This symbiosis will be discussed in further detail in section 1.5.3. Peanut pods enclose one to five seeds that develop underground after flowers are fertilized and elongate to carry ovules into soil (85). This process is known as pegging with developing gynophores generally referred to as pegs. Seeds vary depending on the cultivar, but are generally round to oblong

contained within a straw-colored, brittle hull (82). Peanut plants form a central tap root from which branching lateral roots form.

The type of soil used for peanut cultivation is critical for adequate growth and proper harvesting. Heavily compacted soils, such as those with a high percentage of clay, will negatively impact yield by preventing pods from being excavated during harvest (85). Peanuts are cultivated in well-drained soils, such as sandy or sandy-loam type soils. Peanuts grown in sandy soils, but may require additional water if soil exhibits excessive draining. For optimal growth, peanuts require a soil pH of 6.0 (86). More acidic soil pHs will hinder calcium availability, and can be detrimental to plant growth (85, 86). While there are many nutrients required during peanut growth, calcium is one of the most critical for adequate pod development. Calcium has been found to move very little within plants (87) and in peanuts, has been shown to be required where the fruit is developing for adequate maturation (88, 89). Typically, however, geographical areas most heavily used for peanut cultivation, such as the Southeastern United States, have adequate calcium-soil levels because of their low cation exchange capacity (90-92).

1.5.2 Production and use

In 2014, the USDA reported peanuts were planted across 1.4 million acres in the US, which led to a yield of 5.1 billion pounds of peanuts harvested. The report also estimated that the US peanut production should top 6 billion pounds in 2015 (8). These numbers are dwarfed in comparison to global production, which was reported as 87 billion pounds in 2014 by the Foreign Agricultural Service (FAS) (93). This puts US production at about 7% of the total world production. While still 4th in global production, the US falls behind China (37 billion pounds), India (11 billion pounds), and Nigeria (6.6

billion pounds) in terms of peanut production by weight (93). Local and global consumption of peanuts for its use as a food, oil, and an alternative fuel source will likely continue to grow with the every-growing demand associated with an increasing population.

In terms of demand, the ERS estimated consumption of peanut products in the US was 6.7 pounds per person in 2012 (7). Using population estimates of the time period from the US Census Bureau put total peanut consumption at roughly 2 billion pounds per year (94). As for specific consumption in the US, peanut butter is most highly consumed peanut product at 3.92 pounds per person in 2012, followed by snack peanuts, and peanut candy at 1.28 and 1.22 pounds per person, respectfully (8). Peanut butter consumption in particular has increased 1.21 pounds per person since 1967 (8). Given the current state of peanut consumption in the US and globally, the peanut will continue to be an important crop in the future. This also means that the possible occurrence of a foodborne outbreak could have an enormous negative impact in peanut consumption.

1.6 ***Bradyrhizobium* spp. – organism characteristics**

1.6.1 General characteristics

Like many other organisms residing in the phylum Proteobacteria, the *Bradyrhizobium* genus consists of Gram-negative rods. As a member of the *Rhizobiaceae* family, *Bradyrhizobium* also possesses the ability to fix nitrogen and form a symbiosis with legumes. *Bradyrhizobium* fall into the general class of bacteria known as rhizobia. This general term includes other symbionts of legumes that fix nitrogen including genera such as *Rhizobium*, *Mesorhizobium,* and *Ensifer* (95). *Bradyrhizobium* was only recently

distinguished from the *Rhizobium* genus in 1982 (96). The unique characteristics that warranted the change were the growth characteristics and metabolism of rhizobia in relation to their growth on yeast-mannitol agar (YME). *Bradyrhizobium* grew slower and did not produce acid on YME in comparison to the already delineated *Rhizobium* genus. Thus, the distinct genus, *Bradyrhizobium* was created (96, 97). Similarly, further taxonomic classification was performed to distinguish *Mesorhizobium* (98) and *Sinorhizobium* (currently *Ensifer*) (99) from *Rhizobium*. The organization of rhizobia and subsequently *Bradyrhizobium* will no doubt continue to advance as techniques used for classification provide a clearer picture over time.

1.6.2 Bacterial nitrogen fixation

Nitrogen fixation refers to the process in which atmospheric nitrogen is converted to ammonia. As of now, there are only three known ways to fix atmospheric nitrogen; a bolt of lightning (100, 101), in a chemistry lab via the Haber-Bosch process (100), and through the bacterial enzyme nitrogenase (102). Being relatively inert, molecular nitrogen (N_2) is not usable by plants directly. Therefore, a usable form of nitrogen must be acquired from the soil. This is accomplished by the bacterial conversion of nitrogen in a process called ammonification, in which nitrogen from organic matter is converted into ammonia and is subsequently assimilated by plants through the root system. Legumes take this one step further and develop a symbiosis with nitrogen fixing bacteria to obtain a direct source of nitrogen. Alternatively, there are classes of bacteria, known as diazotrophs, which are able to fix atmospheric nitrogen and convert it to the usable form of ammonia, which can provide usable nitrogen to plants to be used for nucleic and

amino acid synthesis. Nitrogen fixation is restricted to prokaryotes (100), and is one of the key features of symbiotic rhizobia in legumes. The overall reaction scheme for biological nitrogen fixation is as follows:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

The reaction dictates that 8 hydrogen ions are required to convert 1 molecule of nitrogen into 2 ammonia molecules.

The enzyme that catalyzes this reaction, nitrogenase, consists of two major subunits: the iron-containing (Fe protein) exterior subunits and the central molybdenum-iron-containing (MoFe protein) subunit (103). The enzyme must first rely on the reducing power of ferredoxin or flavodoxin to act as the initial donor of electrons to bring the Fe protein to a reduced state (103). With the addition of ATP, this will lead to the reduction of the MoFe protein, which is then capable of reducing atmospheric nitrogen (104). There are several drawbacks to the fixation of nitrogen. The first being the high demand of ATP that is required for the reaction to progress. This number is anywhere between 16 and 24 ATP/N_2 (105-107). The second and more significant issue is that nitrogenase is an oxygen sensitive enzyme that is irreversibly denatured in oxygen rich conditions (108). The other factor that can limit ammonia production is the presence of inhibiting substrates. Carbon monoxide and acetylene are two known substrates that will non-competitively inhibit nitrogenase activity (109, 110). The cell must overcome all of these issues in order for the reduction of atmospheric nitrogen to occur.

1.6.3 Bacterial lifestyle and mechanism of *Bradyrhizobium* infection

There are many reasons for the bacterium and plant to enter a symbiosis. The symbiosis provides a syntrophic environment, where the plant obtains a usable source of

nitrogen and the bacterium has access to nutrients made available through the root system of the plant. The bacterium is additionally provided with a desiccation-free environment and is free from predation. Yet the bacterium still has three pre-programmed lifestyles that will determine if it: 1) enters this symbiosis, 2) remains a free-living soil in the environment, or 3) becomes a parasitic rhizobium incapable of nitrogen fixation yet residing inside plant tissue (95, 111, 112). A cell participating in a symbiosis can have as many as 10^{10} descendants inside a soybean nodule (113, 114). The question then becomes, why, in an advantageous environment such as a root nodule, would non-symbiotic populations of rhizobia be even more prevalent than symbiotic nodule forming rhizobia. The answer possibly surrounds the likelihood that one cell successfully nodulates a plant. In a given soil environment, the odds that a rhizobia cell infects a plant and leads to the development of a nodule is estimated to be one in a million (95). Coupled with the assertion that the offspring of the bacterium may or may not be reproductively viable means that although the organism would produce an extremely high number of descendants, it is very likely that a number of these descendants are incapable of reproduction (95, 115). There are other factors at work here. Predation and the presence of bacteriocins within various environments are difficult to quantify making each environment unique with a distinct set of properties. Another important factor relates to the presence of soil nitrogen, as it has been established that in an environment with excess nitrogen, plants and bacteria forego entering the symbiosis, resulting in a decrease in nodulation (116).

The mechanism by which *Bradyrhizobium* and the plant host form this symbiosis is similar to an infection. Many legumes and rhizobia enter the symbiosis through a

process of root hair curling and infection thread, where plant-microbe signaling causes the root hair of the host to curl and surround a rhizobium cell (125). However, the development of symbiosis between bradyrhizobia and peanut plants follows a different mechanism referred to as crack entry (117). To gain entry to plant tissue, the microsymbiont must penetrate the epidermis of plant tissue via cracks caused by the extension and growth of lateral roots, mainly from the central tap root (117, 118). This results in intracellular spreading and subsequent nodule formation. The relationship between the two organisms is often highly specific, although less so for plants that follow a crack entry infection. Specificity is still held to some degree, as *Bradyrhizobium* is the only genera that will infect peanuts (117). It has also been established that creating artificial cracks in the root system does not lead nodule formation implying that the mechanism followed is specific (117, 119).

Details surrounding the mechanism by which *Bradyrhizobium* inhabits the peanut plant are scarce. There have been, however, a few significant studies that have started to elucidate the interactions in greater detail. The success of nodulation is in part related to exopolysaccharide (EPS) production by the microsymbiont (120, 121). Morgante et al. showed that a *Bradyrhizobium* mutant producing 27% less EPS resulted in a diminished ability to colonize and nodulate peanut plants (121). They also observed that EPS deficient mutants produced nodule-like structures, but very few resulted in nitrogen fixation (121). It has also been established that the generation of reactive oxygen species by plants increases when the plant rhizosphere is exposed to bradyrhizobial Nod factors (NF) (122).

Nod factors are produced by rhizobia in response to plant flavonoids released in the soil. NF will then induce specific responses within the plant some of which are related to gene expression (122, 123). Most of the NF research is related to legumes that follow an infection thread, but Ibañez and Fabra established that NF play an important role inducing cortical cell division in peanuts which is important for nodule development (124). The authors noted that bradyrhizobia with a mutation in the *nodC* gene were able to internalize within peanut tissue, but could not form a nodule, indicating that NF may not be important for early host-symbiont recognition and entry within tissue (124). Thus far the work done on this mechanism has shown the relationship is much more complicated than originally suggested. Additional work must be conducted to further the understanding of this plant-cell symbiosis.

These bacteria undergo metabolic and physiological changes after nodule formation. Once inside a nodule, the resultant changes are so significantly different than native cells, that the cell is referred to as a bacteroid (125). Bacteroids can lose reproductive ability (126-128), essentially rendering them nitrogen-fixing agents for the plant. Several studies have compared the physiology of bacteriods with free-living cultures (129, 130). Overall, many of the differences include the regulation of genes involved in metabolism (130), but proteomic evidence also suggests that bacteroids are subject to reactive-oxygen species (ROS) and osmotic stress (129). Carbon is mainly supplied to the bacteroid in the form of malate and succinate which can feed directly into the tricarboxylic acid (TCA) cycle (131). It also unlikely that a bacteroid can assimilate the nitrogen it fixes, as the capacity to fix nitrogen is unaffected in glutamine synthetase

(GS) and glutamine synthase (GOGAT) mutants (132). This implies that for the bacteroid lifestyle, it is unimportant if the cells are able to assimilate the ammonia they produce.

As a peanut plant requiring a source of nitrogen, the plant can absorb nitrogen directly from the soil, or when nitrogen is limiting in the environment, form a symbiosis with nitrogen fixing *Bradyrhizobium*. Because the nitrogenase enzyme is oxygen sensitive and is irreversibly denatured in oxygen rich conditions (108), nitrogen fixation does not occur under aerobic conditions. To account for this, the plant symbiont produces an oxygen scavenging metalloprotein known as leghemoglobin (133, 134). This protein is present in high levels in the cytoplasm of infected plant cells within the root system and works as a buffer to prevent oxygen concentrations from becoming too high such that nitrogenase is inactivated. This means that the plant is able to supply the bacterium with the proper environment to facilitate nitrogen production. Thus, in nitrogen-limiting environments, the plant-bacteria symbiosis becomes an essential source of nitrogen for the plant.

1.7 **Peanut roasting and peanut butter processing**

Peanut processing begins with planting and subsequent harvesting. Mature peanuts are harvested using specialized machines called peanut inverters that dig into the ground, shake the pods, and finally flip the pods to expose them to the sun. Harvested peanuts have an initial moisture content of roughly 55% (See Results, Section 3.2). After being flipped, the pods are air dried in field before being dried in storage trailers at 35°C to 7 to 10% moisture (135). From there, the peanuts are taken to facilities that will process peanuts for their intended use. For peanuts that are roasted in-shell, peanuts are

first separated from foreign material then washed in wet sand to remove additional debris (135). Peanuts to be roasted after shelling are first separated by size to minimize damage to the kernel when shelling occurs (135). After adjusting for shell size, rollers are then used to remove the kernel from the shell (135). Kernels are then collected and graded based on size and color (135). After this, the peanut kernels are ready for roasting. Typical batch roasting ovens are set to 430°C, with the peanut reaching 160°C (135). The peanuts are roasted for 40 to 60 minutes (135). After cooling, the peanuts are ready for blanching, which can be accomplished using a variety of methods including either dry or wet processes (135). This mild heating step allows the seedcoat to be removed and also allows the cotyledon to be separated from the embryo (135). The embryo is discarded or used in other processes. To ensure quality, the roasted seeds are inspected for color to ensure proper roasting (135). At this point, the peanuts are now ready for milling. During grinding and milling, peanut butter is heated at 70 to 75°C for roughly 20 min. (136, 137). Additional ingredients, such as salt or stabilizers, are added if necessary (135). After briefly cooling the peanut butter in a heat exchanger, the peanut butter is ready for packaging, labeling, and subsequent market distribution.

1.8 **Peanuts as a reservoir for *Salmonella***

1.8.1 Prevalence of *Salmonella* in peanuts

Historically, major outbreaks of *Salmonella* in the food supply were predominantly related to poultry and other meat products. In recent years, an increased number of outbreaks associated with enteric pathogens liked to non-meat products such as fresh produce and dry foods resulted in a need for scientific investigation that would

lead to control the pathogen. The earliest mention of an outbreak of *Salmonella* in a peanut-containing product was in 1994 (138). Since then, a total of seven outbreaks of *Salmonella* caused by peanut products have been reported (139). As such, the nature of the relationship between *Salmonella* and peanuts has being actively studied. Of notable importance is the overall prevalence of the pathogen on raw peanuts brought into a processing facility.

In terms of the presence and prevalence of *Salmonella* in raw peanuts, Calhoun et al. found that *Salmonella* is present in approximately 2.3% of samples (140). This study collected over 900 raw peanut samples, harvested from the three major US peanut growing regions (Southwest, Southeast, and Virginia and the Carolinas). Another study tested over 10,000 samples and found a prevalence of *Salmonella* in peanuts of 0.67% (68 positive out of 10,162) (141). The authors also noted that there was no difference between region samples (eastern versus western), reinforcing the ubiquity of the organism. Reports that have tested multiple nuts reported even less *Salmonella* prevalence. A three-year survey from Australia that included different nuts, only one almond sample tested positive for *Salmonella*. There were 921 total samples, of which 653 were peanut samples (142). While *Salmonella* is widely present in the environment, these studies estimate that the level of *Salmonella* in dry raw peanuts is anywhere between 0 to 2.3%.

1.8.2 Growth and survival of *Salmonella* in peanut products

Because peanut butter is a low water activity food, it was generally regarded as safe from pathogen contamination. However, an epidemiological investigation linking a peanut snack to *Salmonella* illnesses in 1996 opened the possibility that peanuts can be a

vehicle of this pathogenic bacterium and stressed the importance to understand the interaction between *Salmonella* and such products in more detail (4, 138). Overall growth in such products is unlikely, as *Salmonella* cannot grow below a water activity of 0.94 as shown in Table 2 (143).

From Table 2, it is apparent that the ability of *Salmonella* to grow at reduced water activities is fairly similar to other pathogens. This, however, does not mean that the organism cannot survive at water activities that are much lower. The estimated water activity of roasted peanuts and peanut butter are 0.25 to 0.50 and 0.2 to 0.3, respectively (144). For this intrinsic characteristic an outbreak of *Salmonella* caused by peanut butter was thought to be unlikely. Subsequent outbreaks involving peanut butter and other low water activity foods have shown that *Salmonella* can not only remain viable, but also remain virulent. It is for this reason that the behavior of *Salmonella* in low water activity conditions is becoming a popular topic of interest in the scientific community.

The behavior of *Salmonella* in a desiccated environment is a critical piece of information to reduce the impact and likelihood of an outbreak from occurring. Work has been done to study extrinsic factors on the ability of *Salmonella* to remain viable in peanut butter. Of the more common extrinsic factors, temperature was found to be important in long-term storage of peanut butter. The count of a 5 serotype *Salmonella* cocktail in traditional peanut butter stored for 24 weeks decreased 1.6 log CFU/g less when stored at 5°C versus 21°C (9). Similar results were observed by Park et al., who found that *S.* Tennessee levels decreased 0.34 to 1.29 Log CFU/g at 22°C, while only decreasing 0.15 to 0.65 Log CFU/g at 4°C (145).

Table 2: Minimum water activity allowing for growth of selected pathogens. Adapted from (143).

Organism	Minimum growth a_w
Bacillus cereus	0.93
Campylobacter spp.	0.98
Clostridium botulinum type E*	0.97
Clostridium botulinum types A & B**	0.93
Clostridium perfringens	0.94
Enterohemorrhagic *Escherichia coli*	0.95
Listeria monocytogenes	0.92
Salmonella spp.	0.94
Shigella spp.	0.97
Staphylococcus aureus	0.83
Vibrio parahaemolyticus	0.94
Vibrio vulnificus	0.96
Yersinia enterocolitica	0.97

Differences were also observed among the various peanut butter formulations tested. Burnett et al. speculated that *Salmonella* cells will orient towards water droplets, meaning that the size of the droplet within the matrix is a determining factor of pathogen viability (9). This association has not been investigated fully. Overall, *Salmonella* was able to survive in peanut butter for the duration of the study. He et al. studied the effect of carbohydrate content and temperature on the survival of 5 *Salmonella* serovars in peanut butter. They found that a higher carbohydrate content allows for a higher survival rate, but also noted that the surviving organisms had a lower resistance to thermal treatment (146). The authors offered little explanation as to why this occurred, and cited that carbohydrates may offer cellular protection. From a genetic standpoint, little has been

done to understand the mechanism by which *Salmonella* survives in this environment and what genes may give this pathogen an advantage over others.

The other issue related to pathogen survival is the increased difficulty of inactivating it after exposure at low water activities. Under normal high water activity conditions, *Salmonella* is easily inactivated with mild heat treatments (147), but when cells are exposed to low water activity environments cellular inactivation becomes much more difficult. In pecans at 0.52 a_w, a 1-log reduction in viable cell count will take 20 minutes at 120°C (148). In peanut butter, 1-log reduction times were roughly 29 minutes at 71°C and roughly 13 minutes at 90°C (137). Thus, during normal peanut milling conditions (135), a 1-log reduction in the *Salmonella* population would not be achieved during processing. Shachar and Yaron have hypothesized that the colloidal suspension that is peanut butter offers protection to *Salmonella* cells as a central reason explaining the high degree of survival in peanut butter (136). This theory is further reinforced by He et al. who found no significant differences between *Salmonella* and *Escherichia coli* O157:H7 D-values in peanut butter (146). Even upon the removal of the matrix, however, *Salmonella* is still able to gain increased thermal resistance (unpublished data). This means that there is likely an internal mechanism by which *Salmonella* cells acquire this ability, but this has yet to be elucidated.

1.9 **Peanut-related *Salmonella* outbreaks**

As stated earlier in this review, *Salmonella* outbreaks related to peanut-containing products have only been documented over the last 20 years. Since then several recalls due to contaminated products have occurred, as well as several significant outbreaks. Table 2

is a collection of product recalls due to *Salmonella* contamination within the US collected from the FDA since 2010 (149). The table further breaks down this number to show recalls involving nut and nut-containing products. Since 2010, 17.4% of all *Salmonella* implicated product recalls have involved nuts. The high rate of recalls in nuts is due in large part to the increasing frequency of testing done by food companies. Before outbreaks involving nuts occurred, producers did not believe *Salmonella* contamination was a risk in these low water activity foods and did not test for the organism. With three high-profile outbreaks involving *Salmonella* in peanuts over the past 8 years (139), nut companies have been forced to monitor their products and environments, increasing the likelihood that *Salmonella* is found resulting in a product recall. It seems plausible that *Salmonella* has always been present in this environment, and that until recently, food companies simply weren't looking for the pathogen.

Table 3: *Salmonella*-related recalls from nut or nut-containing products**.** Selection criteria includes recalls from nuts, peanuts, cashews, walnuts, macadamia nuts, pine nuts, pistachios, hazelnuts, and pecans

Year	Recalls	Recalls of Nuts	Ratio of total (%)
2010	120	5	4.2
2011	67	5	7.5
2012	138	31	22.5
2013	38	4	10.5
2014	60	5	8.3
2015	77	37	48.1
Total	**500**	**87**	**17.4**

Not only is *Salmonella* in nut products a problem within the food industry, but its presence raises questions about the effectiveness of nut processing. The route of entry for *Salmonella* into a food facility is one of great concern to the food industry. Quality assurance designed specifically to improve food safety programs such as Hazard Analysis Critical Control Point (HACCP), The Global Food Safety Initiative (GFSI), and even laboratory accreditation bodies such as A2LA can help combat this by identifying areas of risk within a food facility. It is still apparent that food processing operations will need to be improved to limit the number of recalled products, especially in nut manufacture.

The first documented case of *Salmonella* associated with peanuts was caused by *S.* Agona in the U. K. in 1994 (4). Since then, there have been seven documented *Salmonella* outbreaks involving peanut products. Table 3 lists these outbreaks in chronological order as they occurred including the product type and the possible route of product contamination. By far the most massive recall involving peanuts was the 2009 outbreak involving *S.* Typhimurium, which sickened 714 people across 46 states in the US. (5). Those illnesses were confirmed cases, and as most individuals do not seek medical attention for gastroenteritis it was estimated that the actual case count was as much as 16 times this number or about 11,000 (1, 150). This outbreak involved the Peanut Corporation of America (PCA), which operated as a distributor of peanut ingredients for other products. The large scope of their distribution system made the removal of contaminated product from consumer markets difficult. This lengthened the outbreak and stressed the importance of analyzing case clusters to possibly identify additional contaminated products (150). Just 2 years prior to this in 2007, 715 individuals were sickened by consuming contaminated peanut butter produced by ConAgra Foods

across 48 states (6). Prior reports of *Salmonella* contamination in final product was reported as early as 2004 (6), meaning that the severity of the outbreak could have been limited if the problem was corrected.

Table 4: List of *Salmonella* outbreaks associated with peanuts or peanut-containing foods.

Year	Serovar implicated	Source	Number of cases	Country	Route of contamination
1994/1995	Agona PT 15	Peanut-flavored savory snack	71	Israel, UK, USA	Unidentified
1996	Mbandaka	Peanut butter	15	Australia	Roasted peanuts
2001	Stanley and Newport	In-shell peanuts	109	Australia, Canada, UK	Imported peanuts
2006	Thompson	Boiled peanuts	100	USA	Peanuts
2006/2007	Tennessee	Peanut butter	715	USA	Unidentified
2008/2009	Typhimurium	Peanut butter	714	USA, Canada	Numerous sources identified
2010	Typhimurium PT170	Peanut/cashew mix	19	Australia	Unidentified
2012	Bredeney	Peanut butter	42	USA	Cross-contamination between raw and finished product
2014	Braenderup	Peanut/Almond butter	6	USA	Unidentified

From Table 3, it is apparent that the ultimate source of *Salmonella* was not identified in all cases, and in those cases in which a food vehicle was identified, the information on where the contamination occurred was unknown. Of particular importance with these outbreaks is how *Salmonella* gains entry to the facility in the first place. The actual entry of *Salmonella* into the PCA facility is unknown, but it has been

suggested that *Salmonella* entered on raw peanuts or was introduced into the facility through areas leaking rain water (150). Concerning the ConAgra Foods outbreak in 2007, the FDA investigation was limited, but found that roasted peanuts were uncovered (6). This was the only major violation and a review of previous product and environmental testing revealed no evidence of contamination in 2005 and 2006. In 2004, however, the company did have product that tested positive for *Salmonella*. Thus, it is likely that the issue was never correctly identified leading to the 2007 outbreak.

From the most recent peanut butter outbreak, the FDA released a series of observations made at Sunland Inc. during the 2012 *S.* Bredeney outbreak. In its report, the FDA documented numerous positive product and environmental samples within the facility. While there were numerous observations made that implicated cross-contamination as the probable cause of the outbreak, the initial introduction of *Salmonella* into the facility remained unidentified (151). Analysis of these three peanut outbreaks has shown the actual entry of *Salmonella* into the facility remains largely unknown. Possibilities include raw peanuts entering the facility contaminated and subsequently contaminate final product, or it could be that some other environmental vector is the source contaminating final product. This makes the possible route of entry for *Salmonella* into a peanut facility an important area of study.

1.10 **Potential risk and impact of future outbreaks**

In 2012, Americans consumed 2 billion pounds of peanuts (7, 94). As long as peanuts are consumed at such high levels, any product contamination with pathogens can result in a severe outbreak. The last major outbreak of *Salmonella* in 2012 cited multiple,

repeated *Salmonella* positive test results (151). *Salmonella* was found in both the environment and in finished product. How this information is handled is critical to minimizing the likelihood of an outbreak.

The overall production scheme of peanuts must be thoroughly examined especially with the knowledge that the milling and grinding of peanuts in peanut butter production is not an effective measure of control against *Salmonella*. To further complicate matters, *Salmonella* is able to survive under low water activity condition, this means that that once the organism inhabits a product or environment its eradication can be difficult. Thus, the importance of environment and product testing programs, along with proper corrective action procedures are critical to ensure that final product is not contaminated. It has been suggested that raw peanuts may enter a facility contaminated with *Salmonella* (150). Whether this occurs in the field via contaminated soil, or occurs during transportation and distribution of raw peanuts remains unknown and warrants additional investigation. The gaps in the distribution chain and prior issues with cross-contamination by multiple companies make it apparent that the risk of a *Salmonella* outbreak with peanuts as a vector is still significant at present day.

CHAPTER 2: MATERIALS AND METHODS

2.1. Bacterial strains and culture preparation

Salmonella enterica serovar Typhimurium ATCC 14028s and *S.* Typhimurium ATCC 14028s with GFP plasmid were used for all experiments of this research. The parent 14028s strain was derived from heart and liver samples of 4-week old chickens in 1960 and has been used as a laboratory strain (152). *S.* Typhimurium GFP possessed an ampicillin resistance cassette as a marker gene. Stock cultures of *S.* Typhimurium 14028s were prepared from a master culture and stored in tryptic soy broth (TSB; Neogen, Inc., East Lansing, MI) containing 20% glycerol at -55°C. *S.* Typhimurium 14028s GFP was supplemented with 100 μg/mL of ampicillin. Cells were resuscitated from stock by plating onto differential tryptic soy agar (dTSA; Neogen, Inc.) containing ferric ammonium citrate (800mg/L), sodium thiosulfate (6.8 g/L), and if necessary, ampicillin at a concentration of 100 μg/mL at 37°C for 24 hours. After incubation, plates were stored at 4°C. Fresh working cultures were prepared every two weeks. *Bradyrhizobium* NC92 was used for plant experiments involving a nodulating endosymbiont. Stock and working cultures were prepared and maintained as described above with two exceptions: Cells were cultured in arabinose-gluconate (AG) broth (153) for 72 hours at 30°C with shaking at 200 RPM. For plates, AG broth was supplemented with 1.5% agar and incubated at 30°C for 1 week.

Inocula were prepared by growing *S.* Typhimurium overnight in TSB at 37°C with shaking at 250 RPM. If the GFP producing strain of *S.* Typhimurium was needed, TSB was supplemented with 100 μg/mL of ampicillin. The inoculum was centrifuged at 4700 x *g* for 10 min and the pellet washed twice in sterile de-ionized (DI) water before resuspension. Cells were resuspended in 10 mL of DI water and further diluted to an

optical density (OD_{600} nm) of 0.300 ± 0.010. The final cellular concentration was then adjusted according to the experiment. *B.* NC92 was prepared by inoculating sterile AG broth and incubating for 72 hours at 30°C, with shaking at 200 RPM. Cells were centrifuged at 7,400 x *g* for 10 min and were washed twice in sterile DI water and diluted to an OD_{600} of 0.150 ± 0.010. The cell suspension was adjusted to reach a concentration of 10^8 CFU/mL.

2.2. Peanut seedpod selection

Two types of peanut pods were acquired for use in this research. Raw green Virginia (GV) peanut pods were purchased from an online retailer (Hardy Farms Peanuts, Hawkinsville, GA) and stored at 4°C until use. For experiments involving dried peanut pods, dry Virginia (DV) peanut pods were purchased dried from an online seed bank (W. Altee Burpee and Co., Warminster, PA). Pods were kept at 25°C until use. The structure of each peanut seed was examined prior to analysis. Only visibly intact seed pods were chosen. The pod was inspected by gently pressing the sides of the peanut together to show the presence of cracks or fissures. If there were visible cracks or other abrasions, the seedpod in was not chosen for examination. Intact seedpods were examined for any colonies exhibiting black growth on dTSA by diluting 11 g of peanut pods 1:10 in 0.85% physiological saline (PS), serially diluting, and spread plating. Concentration was determined by using the colony counting method described in the Bacteriological Analytical Manual (BAM) (154). This method was used to determine the concentration of bacterial populations in all procedures mentioned in this chapter.

2.3. Soil selection and preparation

A Hubbard series (HS) loamy sand soil obtained from Becker, MN was used for pod experiments and whole plant experiments. Potting media (PM) for use in pod experiments was obtained from a local retailer (Schulz Moisture Plus, Infinity Lawn and Garden, Inc., Milan, IL). Each soil was sieved through a 2 mm screen to obtain a uniform particle size. For plant experiments, perlite and HS was mixed at a ratio 1:1 by volume. Both soils were kept in bins at 25°C until sterilization. Both soil types were sterilized by filling autoclavable bins to a depth of 1 to 1.5 cm. The bins were covered in aluminum foil with holes to allow steam penetration. Soil was autoclaved for 60 min at 121°C on two consecutive days (155). Sterility was confirmed by plating a diluted sample on TSA incubated at 37°C for 48 hours.

2.4. Soil pH and water-holding capacity

Soil pH for both soil types was determined by creating slurries of each soil type by adding 10 mL of DI water to 5 g of soil. Five replicates for each soil type were prepared and the pH measured after a two-point calibration (OAKTRON Instruments, Vernon Hills, IL). The average of the five measurements was taken to obtain a final pH for each soil. The water-holding capacity (WHC) was determined following the gravimetric method specified in the Manual of Soil Analysis, Monitoring and Assessing Soil Bioremediation (156). Briefly, dried soil samples in perforated cylinders were submerged overnight in a water-bath. The mass was taken every hour after three hours until little change was observed. Three 5 g samples were weighed into crucibles and placed into a static oven at 105°C for 24 hours. Samples were weighed after 24 hours and

the water-holding capacity determined on a dry weight basis. The experiment was performed three times and the average of the three trials was used to determine the water-holding capacity of each soil.

2.5. Moisture content and water activity determination of soil and peanut seedpods

The initial moisture content (IMC) for both soil and peanuts were determined using the gravimetric method referenced above (156). For soil samples, initial moisture content was determined by adding a specified amount of DI water to each soil type. The mixture was then tested by placing triplicate 5 g samples in a 105°C static oven for 24 hours. After 24 hours, the samples were weighed and the moisture content determined on a dry weight basis. Peanut pods were dried under the same conditions, but samples were prepared by grinding peanut seedpods in a blender before the replicates were weighed. Two independent experiments were performed. Water activity was determined for each IMC using the Aqualab Pawkit (Decagon Devices, Inc., Pullman, WA) following manufacturer's instructions. Three replicates were taken for the measurement of each IMC.

2.6. Persistence of *Salmonella* in soil

Experiments were done to test the persistence and fate of *Salmonella* in each soil type. For each trial, duplicate samples of sterile and non-sterile soil were prepared. HS soil was inoculated with *S.* Typhimurium to a level of 2.5 Log CFU/g and PM inoculated to a level of 4.0 Log CFU/g. Sterile containers of inoculated soil were sealed with

Parafilm M® (Bemis NA, Neenah, WI) and stored at 25°C for the duration of the study. To measure the concentration of *Salmonella* in each soil, duplicate 1 g samples were taken periodically over two weeks. HS soil samples were diluted in 5 mL of PS, before preparing 1:10 serial dilutions. This resulted in a detection limit of 1.7 Log CFU/g. PM samples were diluted in 9 mL of PS before preparing 1:10 serial dilutions, resulting in a 2.0 Log CFU/g detection limit. All dilutions were spread plated on dTSA and Xylose Lysine Deoxycholate (XLD) agar (Neogen Inc.).

2.7. Rate of water absorption by seedpods

To measure the rate of water absorbed by peanut pods over time, individual pods were placed in 8 mL of sterile DI water. Triplicate samples were taken over the 30 hour exposure period 30 hours after exposure and the additional water weight was determined on an analytical balance (Mettler-Toledo, Switzerland). Results were averaged and expressed as the ratio of pod mass gain relative to the initial pod mass.

2.8. Infiltration of *Salmonella* through intact seedpods

2.8.1. Bacterial suspension

In order to assess the diffusion of *Salmonella* cells through intact DV seedpods, a method was developed that prevented contamination of *Salmonella* on the exterior of the seedpod with the interior of the seedpod. Overnight cultures of *S.* Typhimurium 14028s were prepared as described above. The final concentration of the cellular suspension was adjusted to 7 Log CFU/mL with sterile DI water. To understand the effect of concentration on infiltration, the concentration was adjusted depending on the

experiment. To confirm the targeted bacterial count, two 1 mL samples were taken from each suspension, serially diluted in PS and plated on dTSA. The count of *Salmonella* in suspension was also determined by taking 1 mL aliquots periodically over time. The 1 mL samples were diluted in PS and the concentration determined on dTSA.

For suspension trials, a sterile 10 mL container was filled with 8 mL of the *S.* Typhimurium cellular suspension. Polyvinylidene chloride, or plastic wrap, was used as a barrier to suspend an intact seedpod into the suspension without exposing the stem scar. Time, temperature, and concentration were all tested to determine their influence on *Salmonella* internalization. To assess exposure time, seedpods were immersed for 24 hours at 25°C. Samples were taken at intervals to determine and two different exposure periods were tested at 4, 16, 25, 37, and 42°C. Finally, the influence of concentration on *Salmonella* internalization through seedpods was tested at one exposure time with an initial concentration of 2.2, 3.7, 5.7 or 7.4 Log CFU/mL. One control DV pod containing sterile DI water was incubated with three DV pods under all conditions tested. Concentration experiments were performed twice for a total of 2 biological replicates. Experiments testing temperature and time were performed only once. After exposure, pods were again inspected for cracks or fissures. If any deterioration occurred during exposure, the replicate was discarded.

To examine the ability of *Salmonella* to gain entrance to the interior of the peanut shell after exposure, each seedpod was examined for the presence of the bacterium inside the seedpod. The seedpod was placed in a sterile Whirl-Pak® (Uline, Pleasant Prairie, WI) in a manner to create a barrier between the exposed and unexposed portion of the seedpod. The unexposed portion of the seedpod was then removed using a sterile scalpel

and the seeds discarded. The interior of the seedpod was then swabbed for 10 seconds using a sterile cotton-tipped swab hydrated with PS. The swab was then placed in 1 mL of PS and vortexed for 10 seconds to dislodge bacteria adhering to the swab. After swabbing the surface, 1 mL of PS was used to flush out the pod by aspirating the liquid in and out of the peanut pod 10 times. Each of the recovery methods were quantified by serial diluting and plating on dTSA in triplicate. The detection limit was 10 CFU/pod. The results from each method were then added and referred as internal surface recovery (ISR).

2.8.2. Soil

To examine the infiltration of *Salmonella* through intact DV seedpods from soil, both HS and PM soils were tested at different initial soil moisture contents (4, 8, 10, 15, 20, 25, and 30%). Inocula were prepared as described above. The *S.* Typhimurium cellular suspension was adjusted to deliver the appropriate initial moisture content and also a final concentration of 6.5 Log CFU/g in each soil. The concentration of each soil was verified by diluting two 1-g samples in PS. This was followed by preparing serial dilutions and spread plating on dTSA. The concentration of *Salmonella* after the exposure period was also determined in a similar manner. A sterile contained was filled with roughly 15 g of the inoculated soil. DV seedpods were then placed in the soil such that the top portion of the pod containing the stem scar was left unexposed. Sterile spatulas were used to add additional soil to areas of the seedpod that were not properly exposed to soil. Seedpods were exposed to inoculated soil for 72 hours at 25°C. One control DV pod containing sterile PM or HS soil was incubated with three DV pods under all conditions tested. Experiments were repeated three times to obtain three biological replicates. After

exposure, pods were again inspected for cracks or fissures. If any deterioration occurred during exposure, the replicate was discarded. After exposure, ISR was determined as described for the suspension trials.

To compare the effect of drying on *S.* Typhimurium GFP internalization, dry green Virginia (DGV) seedpods were prepared in a similar method used in the peanut industry by drying GV pods in a dehydrating oven for 24 hours at 40°C. The moisture content of GV and DGV pods was determined as described previously. To reduce the effect of background microflora observed within GV peanut pods, *S.* Typhimurium GFP was used to examine infiltration. To ensure that the GFP containing strain did not differ in its ability to infiltrate a seedpod, DV pods were tested as described above with *S.* Typhimurium GFP. Infiltration of *S.* Typhimurium GFP was tested in GV and DGV pods at 20% and 30% initial moisture content. Samples were prepared and tested under the same conditions described above to determine ISR with the exception that dTSA was amended with 100 μg/mL of ampicillin. In addition to the presence of black colonies, UV light at 354 nm was also used to confirm the presence of fluorescing *S.* Typhimurium colonies on dTSA. To understand the degree of plasmid loss in the system by *S.* Typhimurium GFP, the initial and final concentrations of *S.* Typhimurium GFP within HS soil were analyzed by spread plating on plates with and without ampicillin. Three DV and three DGV pods were exposed for 72 hours at 25°C. Experiments were repeated three times to obtain three biological replicates. After exposure, pods were again inspected for cracks or fissures. If any deterioration occurred during exposure, the replicate was discarded. After exposure, ISR was determined as described for suspension trials with the exception that dTSA was supplemented with 100 μg/mL of ampicillin.

2.9. Seed preparation and plant growth conditions

To characterize the internalization of *S.* Typhimurium in plants through soils, seeds were first sterilized prior to sowing. DV seedpods were shelled and soaked in DI water for two minutes to loosen the seedcoat. The seedcoat was removed and the seeds were transferred to a sterile 50 mL centrifuge tube containing 70% ethanol. The seeds were vortexed for 10 seconds and transferred to a 10% bleach solution and vortexed for 30 seconds. A total of three rinses in 10% bleach were performed. Residual sodium hypochlorite was removed by rinsing the seeds 3 times using sterile DI water. Sterilization was confirmed by placing a sterile seed in molten TSA. After the agar solidified, plates were incubated at 37°C for 48 hours.

Plants were grown in an incubator modified to accommodate plant growth. Plants were grown using full spectrum light at a distance of roughly 2 feet with 16 hour photoperiods. The temperature and humidity were kept constant at 26°C and 65% RH. The conditions were monitored daily. Plants were watered carefully on the top of soil with 35 mL of sterile DI water every 48 hours.

2.10. Internalization of *Salmonella* through soil in growing plants

To study the internalization of *Salmonella* into peanut plants through the root system, peanut seeds were grown under the conditions described in previous sections. Sterile soil was inoculated with *S.* Typhimurium to a level of 6 Log CFU/g. The concentration was tested at each time point using dTSA by taking two 1 g samples, serially diluting in PS, and spread plating. Two plants were harvested at 14, 21, and 28

days. The experiment was repeated two times to obtain a total of three biological replicates.

To study the effects of the natural peanut symbiont, *Bradyrhizobium*, on internalization, seeds were grown as described previously except that sterile seeds were dipped in an 8 Log CFU/mL suspension of *Bradyrhizobium* NC92 for two minutes prior to sowing. To prevent contamination, plants containing *B.* NC92 were kept on the opposite side of the modified incubator and always kept below the non-treated plants. Plants were harvested and inspected for nodulation upon harvesting at 21, 28, and 35 days. One plant inoculated with *B.* NC92 and one plant not inoculated with *B.* NC92 was harvested at each time point. The experiment was repeated one time to obtain two biological replicates.

Each plant was surface sterilized to ensure the removal of bacteria prior to analysis. This procedure was adapted from Zhe et al. (157). Whole plants were harvested and the root system freed of soil by rinsing with DI water. Plants were then submerged in 70% ethanol for 10 seconds, followed by immersion in 5% bleach for 30 min. Whole plants were further sterilized by exposure to UV light at 190 to 290 nm for 30 minutes on each side. The sterilization procedure was verified by overlaying dTSA on portion of stem, leaf, and root tissue. The tissue was incubated at 37°C for 48 hours. The test was discarded if the presence of black bacterial growth was observed.

To determine the localization of *Salmonella* within host tissue, plant leaves, roots, and stems were aseptically separated and tested individually. Each leaf, root, and stem sample was diluted 1 to 5 by mass in PS and ground using a mortar and pestle. This resulted in a detection limit in 50 CFU/g of plant tissue tested. After grinding, samples

were serially diluted in PS and plated using dTSA in duplicate. The final concentration of *Salmonella* within host tissue was then calculated on a per gram basis as described previously.

2.11. Statistical analysis

All statistical calculations were performed using the R statistical package (R Version 3.1.2, The R Foundation, Wien, AU). To understand the variation of ISR at different IMC for HS soil, a global analysis of variance (ANOVA) assuming equal variance was performed. If differences were observed, Tukey's comparison of means test was carried out to determine where differences lie. Two-sample Student's t-tests assuming equal variance were conducted to compare the ISR of DGV and DV pods with $\alpha = 0.05$. For analysis of plant localization patterns, ANOVA and Tukey's comparison of means test were performed similar to that described above for seedpod internalization experiments. To compare the effect of the peanut symbiont, *Bradyrhizobium*, on *S.* Typhimurium internalization, a two-sample Students's t-test assuming equal variance was carried out with $\alpha = 0.05$.

CHAPTER 3: RESULTS

3.1 Internalization of *S.* Typhimurium in peanut seedpods through suspension

Several experiments were performed to understand the factors affecting the infiltration of *S.* Typhimurium within DV seedpods. Before examination, the bacterial population during seedpod exposure was measured over 24 hours. As shown in Table 4, the suspension of *S.* Typhimurium during seedpod exposure increased 0.64 Log CFU/mL after 24 hours. The overall suspension concentration ranged from 7.26 to 7.90 Log CFU/mL throughout the time points tested. The variation was low between the three replicates, but was higher at all time points when compared with time zero.

Table 5: Concentration of *S.* Typhimurium suspension over time during seedpod exposure.

Time	Concentration	Standard deviation	Difference from initial
	Log CFU/mL		
0	7.26	0.02	-
4	7.31	0.09	0.05
22	7.90	0.13	0.64
24	7.90	0.10	0.64

To determine the effects of temperature on internalization, two time points were chosen. *S.* Typhimurium was detectable on the interior of DV seedpods under every temperature (Figure 1). The difference between the 4 and 22 hour exposure was more than 1 Log CFU/pod at all temperatures, with the exception of 16°C when the average ISR at 4 h was 0.5 Log CFU/pod greater than at 22 h. A decrease in ISR of 1.25 Log CFU/pod at 4 h and 0.77 Log CFU/pod for the 22 h exposure were also determined when the temperature was increased from 37 to 42°C.

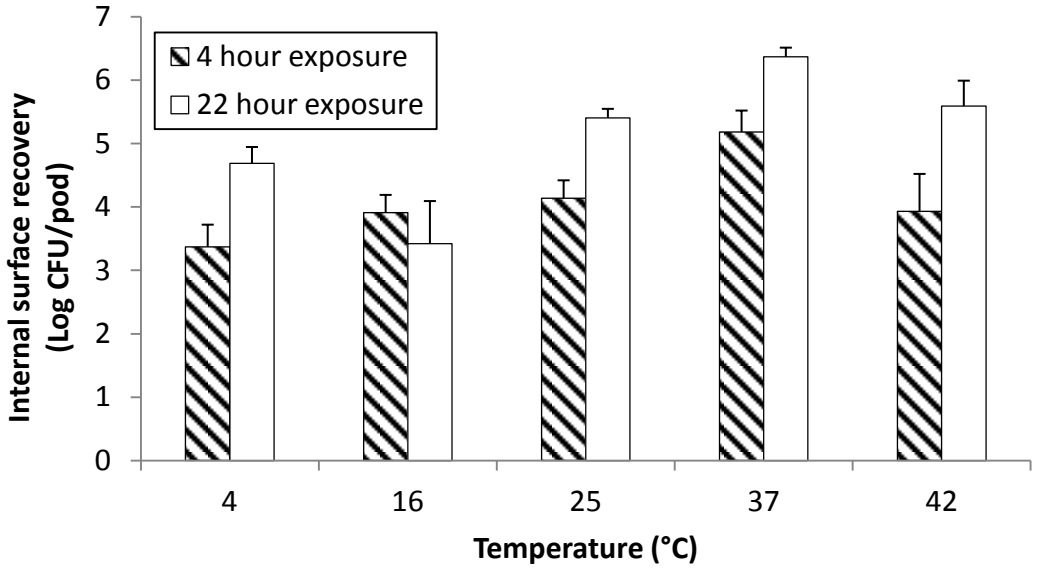

Figure 1: Effect of temperature on the rate of bacterial infiltration into seedpods when immersed into *S.* Typhimurium water suspensions. Seedpods were submerged in a 7 Log CFU/mL suspension. Error bars represent standard deviation of the mean.

DV pods were exposed at eight different time points at 25°C to assess the effect of time on internalization. *S.* Typhimurium was recovered on the interior surface of DV pods at all exposure times (Figure 2). The overall trend of these experiments was that at longer exposure times the larger counts were determined. The highest recovery of *S.* Typhimurium from the interior surface was observed at 22 h at a level of 6.10 Log CFU/pod, while the lowest recovery was measured at 1h exposure (2.7 Log CFU/pod). However there was extensive variation at each time point and the difference between the 0.5 h and 24 h periods was only 1.2 Log CFU/pod. The standard deviation during each exposure period was relatively high ranging from as little as 0.34 Log CFU/pod for the 4 hour exposure period to 1.64 Log CFU/pod for the 16 hour exposure period.

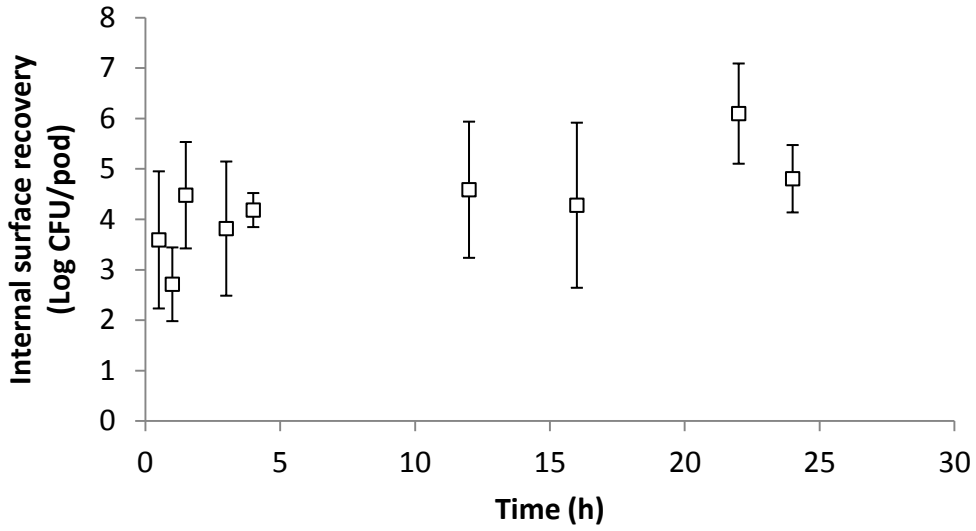

Figure 2: Effect of time on *S.* Typhimurium infiltration of seedpods when exposed to a cellular suspension. Seedpods were submerged in a 7 Log CFU/mL suspension. Error bars represent standard deviation of the mean.

The results of the cell concentration on the recovery of *S.* Typhimurium inside DV peanut pods are summarized in Figure 3. DV pods were exposed for 2 h at 25°C. No *S.* Typhimurium cells were detected on the interior of seedpods at 2.2 Log CFU/mL. The detection limit was 1.0 Log CFU/pod. All other treatment groups had detectable levels of *S.* Typhimurium on the interior surface. No difference was observed among the 3 other time concentration levels tested ($p > 0.05$). The average ISR for the 2-h exposure at 7.4 Log CFU/mL was 2.1 Log CFU/pod, which was 2.38 Log CFU/pod lower than that of the 1.5-h exposure used to understand the effect of time on *S.* Typhimurium internalization. The same conditions were used for both tests.

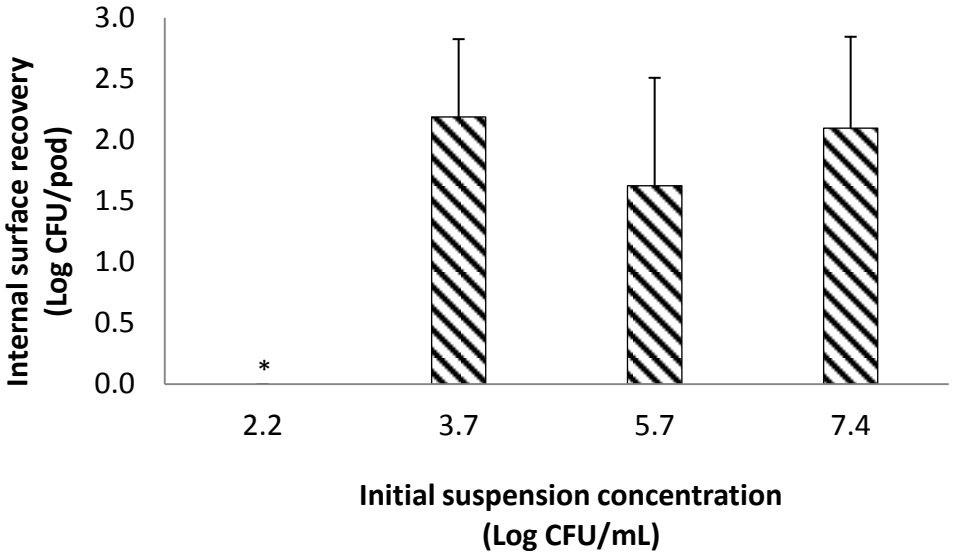

Figure 3: Effect of *S.* Typhimurium concentration in water suspension on the internal recovery inside seedpod. The limit of detection was 1 Log CFU/pod. (**S.* Typhimurium was not detected). Error bars represent standard deviation of the mean.

3.2 Internalization of *S.* Typhimurium into peanut seedpods from soil

Soil types were characterized by measuring pH, water activity, and water holding capacity (WHC). The soil pH of PM and HS soil was determined to be 6.66, and 5.04, respectively. Water activity was also examined and determined across a range of initial soil moisture contents for HS soil only. HS soil dried to a level of 1% moisture had a water activity of 0.75. The addition of 1 (5.8% moisture), 3 (15.4% moisture), 6 (30.1% moisture), and 9 mL of DI water to 20 grams of HS soil all had statistically similar water activities at 0.93 ± 0.01. Finally, WHC was determined for both PM and HS soil. The WHC of PM was determined to be 8.2 times higher than the WHC of HS soil. The average values for PM and HS soil were 202 ± 10% and 25 ± 4%, respectively.

In order to expose seedpods to soil with known initial moisture content (IMC), the amount of water added to both soil types was correlated to the moisture content. Results are summarized in Figure 4. Linear R^2 values were 0.98 and 0.99 for PM and HS soil, respectively. The moisture content calculated based on the amount of DI water added to soil differed from the empirically determined moisture content by 1.7 and 0.5% on average for PM and HS soil, respectively.

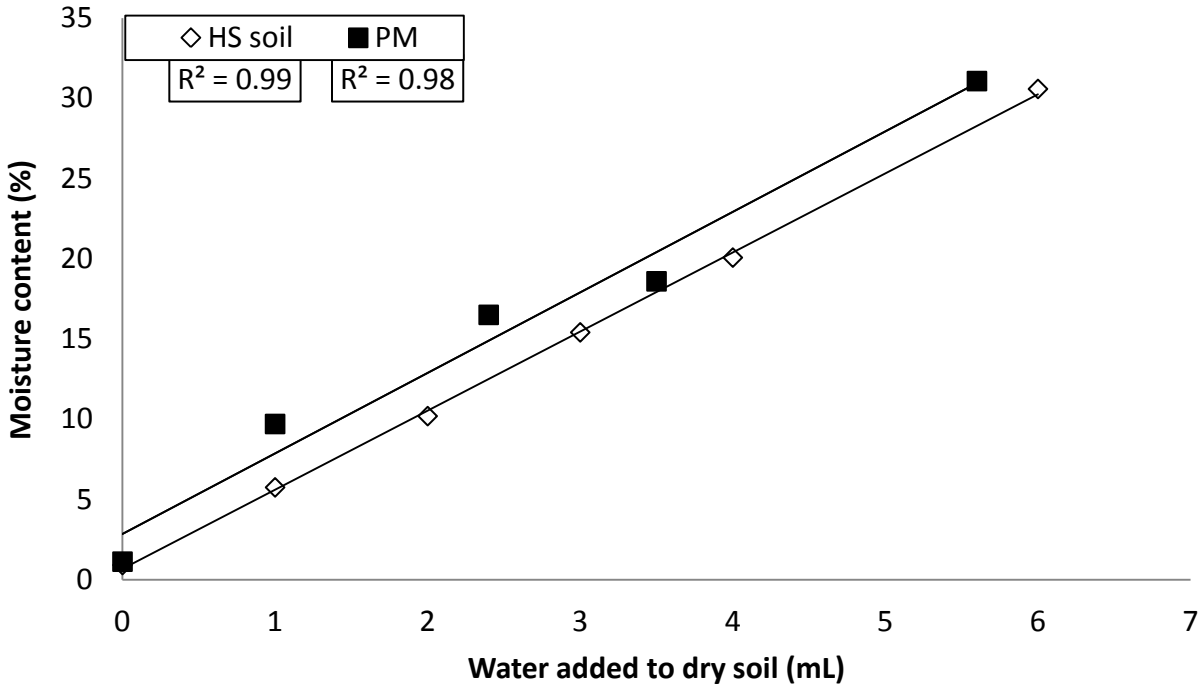

Figure 4: Water added to dried soil and the effect on soil moisture content.

The behavior of the *S.* Typhimurium population in soil was also examined over time. Sterile HS soil was the only sample where *S.* Typhimurium was capable of growing. *S.* Typhimurium increased four orders of magnitude to a level of 6.4 Log CFU/g after 11 days (Figure 5). *S.* Typhimurium was undetectable at day 6 in nonsterile HS soil,

and at day 5 in both sterile and nonsterile PM. The decrease in *S.* Typhimurium concentration observed in PM was at least 2 Log CFU/g over the first 5 days, while the decrease in concentration observed in HS soil was at least 0.54 Log CFU/g over the first 6 days. The concentration of *S.* Typhimurium in sterile HS soil also showed a high degree of variation between replicates at 5 days post inoculation with a standard deviation of at 1.55 Log CFU/g. Later testing points exhibited a standard deviation roughly one Log CFU/g lower at 0.50 Log CFU/g.

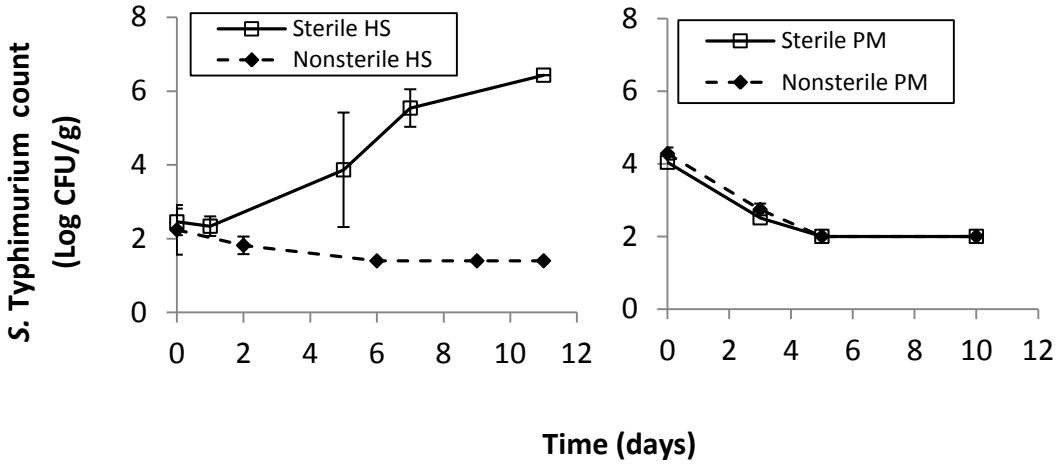

Figure 5: *S.* Typhimurium count in sterile and nonsterile soil over time. Samples were prepared at an initial moisture content of 20%. The detection limit for the HS soil was 1.7 Log CFU/g and 2.0 Log CFU/g for PM soil. Error bars represent standard deviation of the mean.

Using the data from Figure 4, *S.* Typhimurium inocula were prepared to deliver the correct amount of cells and DI water to achieve the proper initial moisture content of soil during seedpod exposure. The initial and final cell count in both soil types was

determined for each experiment (Figures A.1 and A.2 of the Appendix). The concentration of *S.* Typhimurium recovered from PM soil declined significantly when the IMC was below 20%. The average decrease observed at these 4 IMCs was 1.8 Log CFU/g. No differences were observed when IMC was 20, 25, and 30%. Data was also collected for soil exposure when IMC was 40% for PM soil. The concentration of *S.* Typhimurium recovered from PM soil with an IMC of 40% increased significantly from the initial concentration. The average increase was 0.51 Log CFU/g. In contrast, significant differences in HS soil concentrations were not observed for 8 and 10% IMC. Not enough final concentration data was collected to obtain *p*-values for 4, 15, and 20% IMC. Decreases of 0.77 Log CFU/g were observed for IMC below 20%. The difference between initial and final concentration was similar for 20, 25, and 30% IMC.

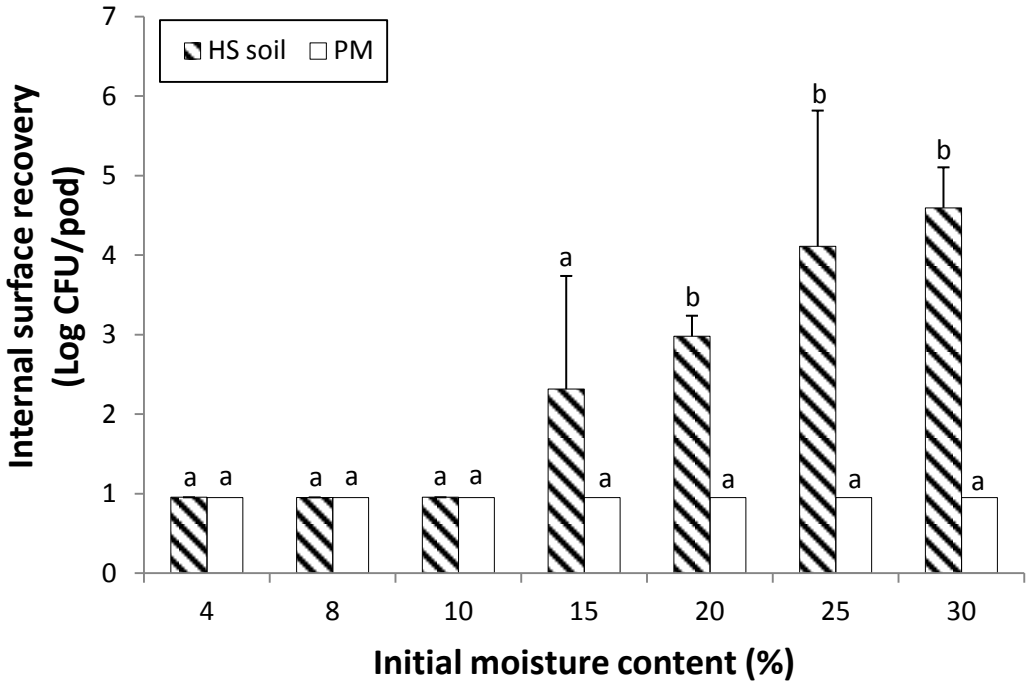

Figure 6: Effect of soil type on the internalized *S.* Typhimurium population in peanut pods at different soil initial moisture contents. Difference in mean values are

significantly different if letters above each bar are not the same (α = 0.05). Error bars represent standard deviation of the mean.

The infiltration of *S.* Typhimurium in DV seedpods upon exposure to both soil types is summarized in Figure 6. No *S.* Typhimurium was detected (detection limit was 1.0 Log CFU/pod) on the interior of DV seedpods when inoculated in PM soil, regardless of IMC. Data for PM at an IMC of 40% was also collected with similar results. No *S.* Typhimurium cells were recovered on the interior surface of DV pods when exposed to inoculated HS soil with an IMC below 15%. As the IMC was increased from 15%, the average amount of *S.* Typhimurium recovered increased from 2.31 Log CFU/pod at 15% IMC to 4.59 Log CFU/pod at 30% IMC. Significant differences between PM and HS soil were observed for every IMC above 15%. *p*-values from Tukey's multiple comparison of means analysis of HS soil IMC is summarized in Table 5. Significant differences were observed for 25 (mean was 4.11 Log CFU/pod) and 30% (mean was 4.59 Log CFU/pod) IMC when compared to when IMC was below 10% (mean was < 1.0 Log CFU/pod). The comparison between 30 and 15% (mean was 2.31 Log CFU/pod) IMC was also moderately significant (p = 0.03). This was the only comparison yielding significance when *S.* Typhimurium was recovered from the interior surface of the pod.

Table 6: *p*-values from Tukey's multiple comparison of means for varying initial moisture content in HS soil. Values below 0.05 are in bold.

Initial moisture	Initial moisture content

content	4%	8%	10%	15%	20%	25%
8%	1.000	-	-	-	-	-
10%	1.000	1.000	-	-	-	-
15%	0.454	0.454	0.454	-	-	-
20%	0.143	0.143	0.143	0.954	-	-
25%	**0.008**	**0.008**	**0.008**	0.183	0.713	-
30%	**0.001**	**0.001**	**0.001**	**0.031**	0.275	0.990

Figure 7 expresses the change in pod mass when immersed into DI water. After only 1 h of exposure, DV pods absorbed an average of 8.5% more water by mass than GV pods. This means that DV pods absorbed 3 times more water than GV pods when compared by weight. The mean difference between DV and GV pods was 12 ± 3% over the course of the experiment. Two-sample t-tests were performed at all time points. The percent mass gained of DV pods was significantly higher ($p < 0.05$) than GV pods at every time point except at 30 h where p was 0.12. The average moisture content of DV pods was determined to be 4%, while GV pods had an average moisture content of 55%. To compare dry pods to green pods, GV pods were dried to an average of 10% moisture.

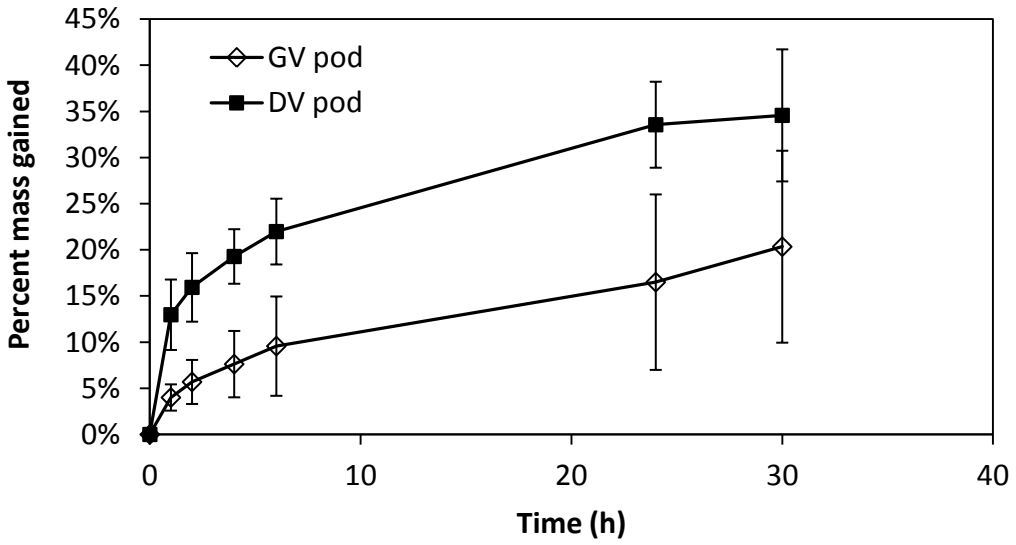

Figure 7: Increase in weight by peanut pods after immersion in sterile water at 25°C. Values calculated relative to the initial mass of the peanut. Error bars represent standard deviation of the mean.

The internalization of *Salmonella* into GV pods was first attempted using wild-type *S.* Typhimurium as performed for DV seedpods. However, the large counts of background microflora recovered (> 5.5 Log CFU/pod) prevented the differentiation of *S.* Typhimurium from other bacteria. Thus, GV pods were examined using the *S.* Typhimurium GFP strain. dTSA media with ampicillin reduced the number of background microorganisms to 3.3 Log CFU/pod, which allowed for the differentiation of *S.* Typhimurium on dTSA. The recovery of internalized *S.* Typhimurium GFP was compared to wild-type using DV pods. *S.* Typhimurium GFP was recovered at 4.8 Log CFU/pod in DV pods at 30% IMC. This was 0.3 Log CFU/pod greater than the average recovery for *S.* Typhimurium without the plasmid, and was within the standard deviation for that dataset (0.51 Log CFU/pod). To further characterize losses of the plasmid, the *S.* Typhimurium GFP concentration in HS soil was measured before and after the exposure period with and without antibiotic stress on plating media. The average difference between the initial concentration with and without ampicillin was 0.02 Log CFU/g. Similarly, the difference between the final concentration with and without ampicillin was 0.07 Log CFU/g.

S. Typhimurium GFP was detected inside GV and DGV pods, although recovery was lower in GV pods. Average recovery for DGV pods at 30% IMC was 3.6 Log CFU/pod, but was only 1.4 Log CFU/pod for GV pods held under the same conditions. Internal recovery of *S.* Typhimurium GFP was 1.2 and 2.3 Log CFU/pod lower in GV

pods than DGV pods. This difference was only significant when IMC was 30%. The standard deviation for DGV pods was 0.89 Log CFU/pod at 20% IMC and 0.37 Log CFU/pod at 30% IMC.

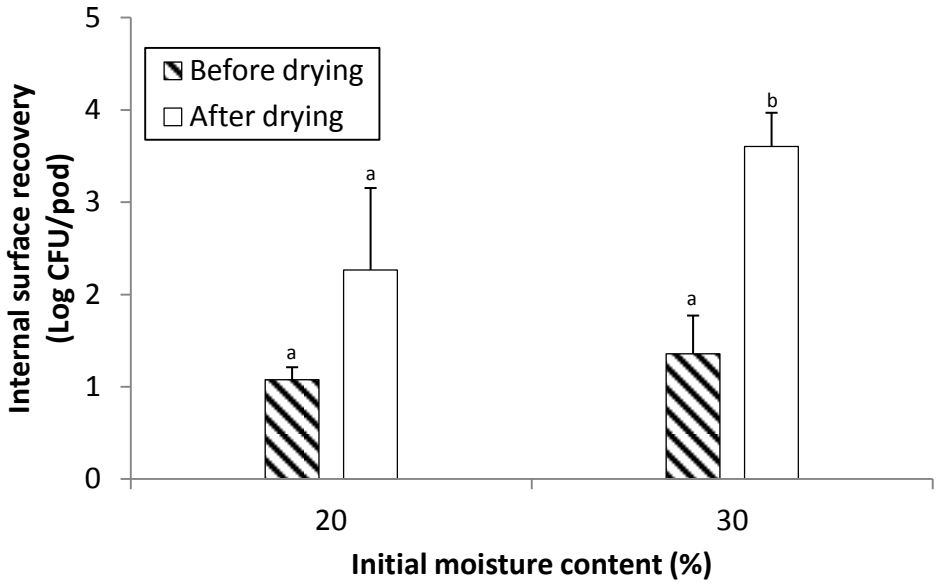

Figure 8: The effect of drying green pods on the internalization of *S.* Typhimurium. Difference in mean values are significantly different if letters above each bar are not the same ($\alpha = 0.05$). Error bars represent standard deviation of the mean.

3.3 Internalization of *S.* Typhimurium in growing peanut plants

3.3.1 Internalization and localization of *S.* Typhimurium within plant tissue.

The population of *S.* Typhimurium in HS soil was determined during the growth period of the plants (Figure 9). The average *S.* Typhimurium concentration decreased 1 Log CFU/g after 35 days. The initial concentration was 6.36 Log CFU/g, which increased at day 2, but then steadily declined to a final concentration of 5.33 Log CFU/g. The

largest reduction in the *S.* Typhimurium concentration was observed at 14 and 21 days, when the concentration decreased 0.5 Log CFU/g.

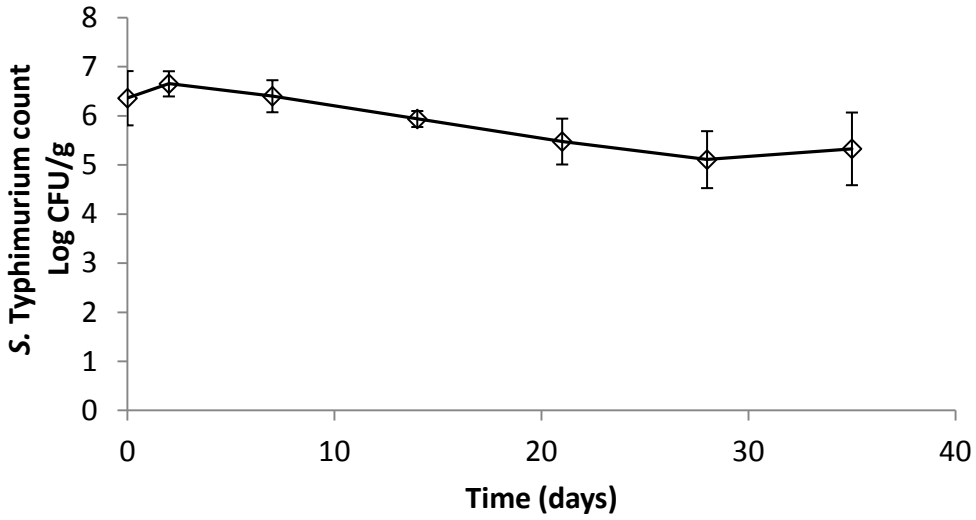

Figure 9: *S.* Typhimurium population in HS soil while plants were grown. Error bars represent standard deviation of the mean.

Internalized *S.* Typhimurium was recovered at a level of 3.5 Log CFU/g from stem tissue, 2.6 Log CFU/g from root tissue, and 1.7 Log CFU/g from leaf tissue (Figure 10). The recovery of *S.* Typhimurium from the stem tissue was 0.9 and 1.8 Log CFU/g greater than the root and leaf tissue, respectively. *S.* Typhimurium internalized in stem tissue at significantly greater levels than both root and leaf tissue at day 14 and only leaf tissue at day 21. No significant differences were observed at day 28 between any tissue. These differences are also reflected in the number of plants with detectable *S.* Typhimurium for each plant section. *S.* Typhimurium was detected at the highest frequency in stem samples at 88% (15/17). In roots and leaf samples *Salmonella* cells

were detected in 76 (13/17) and 35% (6/17), respectively. Plants examined for the third trial on day 28 did not contain any internalized S. Typhimurium, greatly increasing the variability of the data set. This is reflected in the standard deviation for 28 day stem tissue (1.3 Log CFU/g), which is 0.8 Log CFU/g greater than what was observed for 14 and 21 day stem tissue (0.5 Log CFU/g).

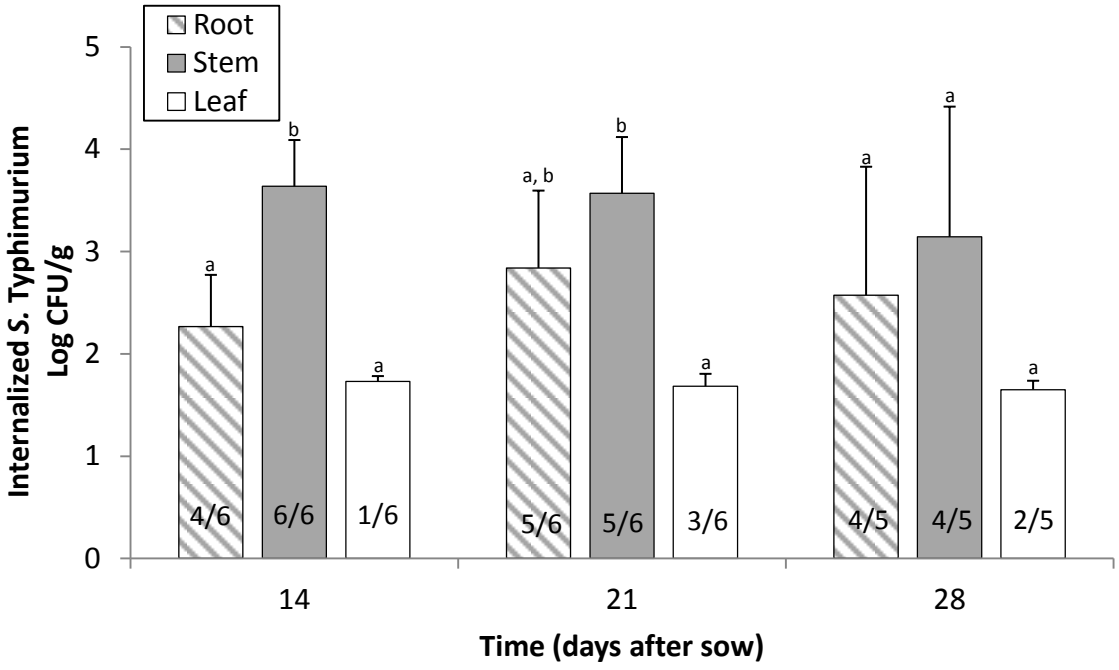

Figure 10: Recovery of internalized *S.* Typhimurium at 14, 21, and 28 day after sowing. Fractions in bars represent the number of plants in which *S.* Typhimurium was recovered. Difference in mean values are significantly different if letters above each bar are not the same (α = 0.05). Error bars represent standard deviation of the mean.

Confidence intervals (95%) for differences in mean internalized S. Typhimurium are presented in Figure 11 as a forest plot. Differences in localization between each plant section are significant when the confidence interval did not include zero. The simultaneous comparisons confirm that differences between the level of internalized S. Typhimurium in stem and leaf samples at day 14, stem and root samples at day 14, and stem and leaf samples at day 21 were significant. The difference in tail length also increased 2.6 times from day 14 to day 28, which reinforces the high degree of sample variability for 28-day samples.

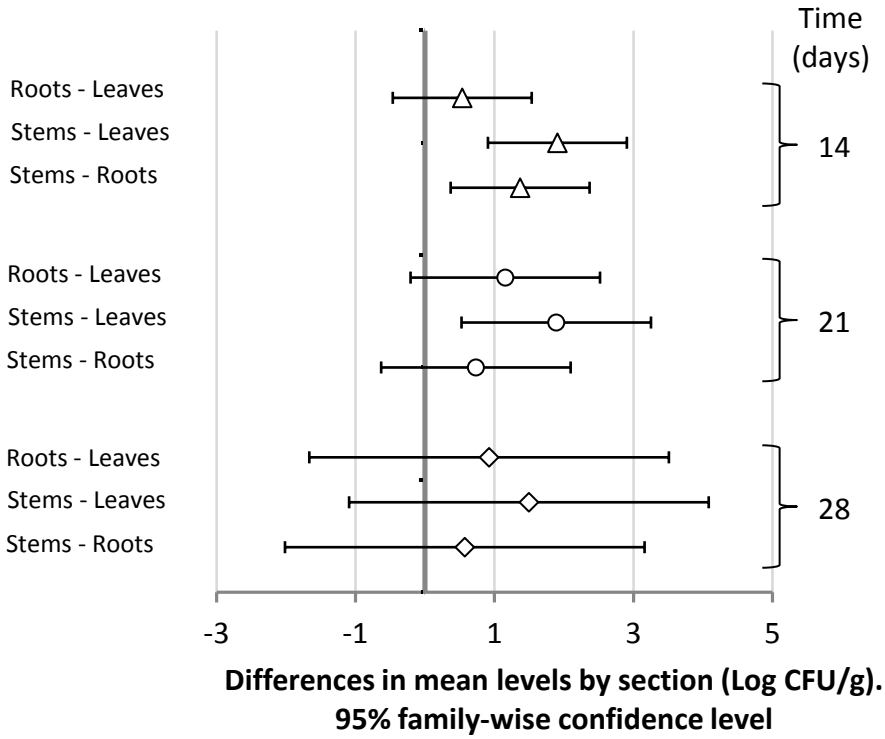

Figure 11: Differences in mean internalization count by plant sections. Tukey's comparison of means was used to test across each plant section. Statistically significant results are indicated by confidence intervals not including zero.

3.3.2 The effect of the *Bradyrhizobium* on *S.* Typhimurium internalization

Peanut plants were grown with *Bradyrhizobium* NC92 to examine the nodulating symbiont's effect on the ability of *S.* Typhimurium to gain entry into plant tissues. In all trials, the recovery of internalized *S.* Typhimurium was lower when peanuts had been dip-inoculated in *B.* NC92. This difference, however, was not significant for any tissue type at any time point examined. The average difference in internalization of *S.* Typhimurium for all plants tested was 1.1 Log CFU/g for stem samples, 0.7 Log CFU/g for root samples, and 0.6 Log CFU/g for leaf samples (Figure 12). *S.* Typhimurium was not recovered from leaf tissue (0/6) for plants inoculated with *B.* NC92, but was recovered in 33% (2/6) leaf samples. Detection was also less frequent in root samples for those plants inoculated with *B.* NC92, where 50% (3/6) of samples contained internalized *S.* Typhimurium, compared to 67% (4/6) root samples contained the pathogen in plants that were not inoculated with *B.* NC92. Internalized *S.* Typhimurium was recovered from all stem samples (6/6) in plants inoculated with and without *B.* NC92. The localization of *S.* Typhimurium for peanuts inoculated with *B.* NC92 is similar to that observed in Figure 10 as the *S.* Typhimurium count was greater in stem samples as compared to roots and leaf samples.

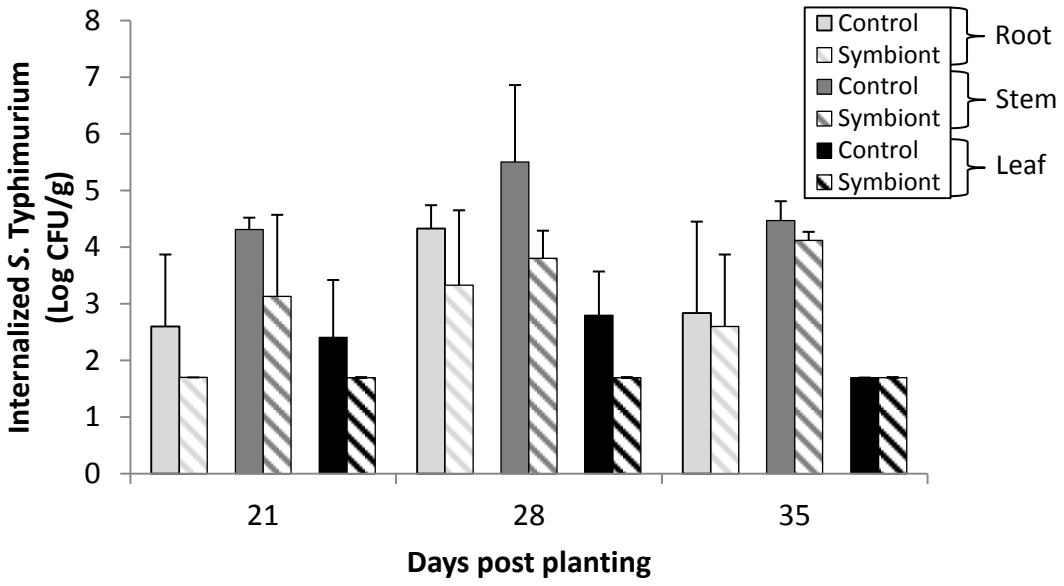

Figure 12: Effect of inoculation of peanut seeds with *B.* NC92 on internalization of *S.* Typhimurium present in soil into peanut plants. Error bars represent standard deviation of the mean.

CHAPTER 4: DISCUSSION

4.1 Internalization of *S.* Typhimurium through intact seedpods

One of the findings obtained from this research was that intact peanut seedpods were susceptible to *S.* Typhimurium infiltration. Similar results have been reported in *Salmonella* infiltration studies performed with pecans (158), almonds (159), and walnuts (160). Beuchat and Mann, who studied *Salmonella* in pecans reported the infiltration of *Salmonella* within the nuts (158). This observation was established as a relation of the total amount of *Salmonella* recovered from the exposure of whole pecans and not a relation of *Salmonella* recovered from the interior surface of the pecan shell. Our results support the internalization of *Salmonella* into peanut shells and also establish the notion that *Salmonella* is capable of internalization in peanut seedpods. Moreover, internalized *S.* Typhimurium occurred rapidly after exposure in as little as 0.5 h from a 7 Log CFU/mL suspension. Given the proper conditions *S.* Typhimurium is also capable of contaminating the interior of DV seedpods at high levels (over 6 Log CFU/pod). It was not determined if *S.* Typhimurium was actively growing upon gaining entry to seedpods. This may be a likely scenario given that *S.* Eneritidis is capable of growth in almond hulls soaked with water (159, 161). Thus, it can't be ruled out that in optimal conditions, the pathogen could be able to grow and reach impactful levels within DV seedpods.

Danyluk, Brandl, and Harris revealed in their study of almonds that non-motile *S.* Typhimurium migrates through almond hulls at the same rate as motile *S.* Typhimurium (159). That observation and the results of the current study supported the idea that *Salmonella* was able to migrate through peanut seedpods in a passive rather than active manner. It can be speculated that the ability of the pods to absorb water may have played an important role in the pathogen gaining entry to the interior surface. DV seedpods

absorbed 3 times more water than GV pods in the first hour of exposure (Figure 7) and *S.* Typhimurium was recovered from the interior surface at a level of 2.7 Log CFU/pod. While a direct comparison between GV and DV pods was not made using *S.* Typhimurium suspensions, *S.* Typhimurium recovery was significantly greater in DV pods than GV pods when exposed to inoculated HS soil. A discussion of *S.* Typhimurium internalization in peanuts from soil is addressed below.

Analysis of the variables tested for suspension experiments did not yield significantly different results. It is important to note that, while no significance was observed between results, there were some overall trends. When the concentration of the initial suspension was decreased to 2.2 Log CFU/mL, no *S.* Typhimurium was recovered from the interior of the seedpod. This was the only concentration in which *S.* Typhimurium was not recovered from the internal surface. Likely, the measurement technique played a role in this, as the detection limit of 1 Log CFU/pod is high given the inoculum level. No differences in the amount of internalized cells were observed at other concentrations. The degree of variability between results at other concentrations makes the association between concentration and internally recovered *S.* Typhimurium difficult to quantify. Qualitative detection of the pathogen would have aided in adding clarity to the association as the detection limit would have been assumed to be 1 *S.* Typhimurium cell/pod.

The amount of cells recovered at higher temperatures was greater than at lower temperatures (Figure 1). The amount of water absorbed by pods and the metabolic activity of cells may help explain the differences observed. Initial and final masses of the peanut seedpods tested did not follow any pattern (see appendix), thus it is unlikely that

the temperature significantly impacted the amount of water absorbed by DV pods. If we take into account that cells are capable of growth and actively dividing while passively diffusing through the peanut shell then the trend observed when temperature is changed can be partially explained. It is likely that a combination of factors contribute to the high degree of internalization observed at elevated temperatures. Moisture content and the porosity of the seedpod are two additional possibilities.

Salmonella contamination of the interior of peanut seedpods through soil was examined to provide a more realistic model of how *Salmonella* may be transmitted in a field or after drying during transportation and storage. The current study is the first to investigate this relationship in peanuts. The effect of soil water activity was initially targeted as a variable of interest in *Salmonella* internalization from soil, but was abandoned because the water activity did not significantly change from 5% to 30% moisture content in HS soil. Dry soil was determined to have a water activity of 0.75, and increasing the moisture content to 5% resulted in a soil water activity of 0.94. *Salmonella* is capable of growth at this water activity and no internalization was observed when initial moisture content (IMC) was below 15% indicating that soil water activity had little impact on the internalization of *S.* Typhimurium.

Overall results indicated that *S.* Typhimurium internalization of peanut pods through soil was moderately dependent on the IMC of HS soil. Global analysis of variance results identified differences between the internalization of *S.* Typhimurium at different soil IMC. Internalized *S.* Typhimurium was not detected under dryer conditions (IMC below 15%) even though a large portion of the initial inoculum remained viable. Moreover, *S.* Typhimurium internalized more readily when IMC was higher (Figure 6

and Table 5). The absorption of water by seedpods and subsequent diffusion of cells under these conditions continues to be the most likely scenario allowing *S.* Typhimurium to gain entry into seedpods. Measurement of the amount of water absorbed by pods during exposure to HS soil could not be accomplished, because soil attached to the peanut pod surface could not be adequately removed without damaging the pod.

Two types of soil matrices, Hubbard series (HS) soil and potting media (PM), were compared to assess the impact of soil on *Salmonella* internalization. *S.* Typhimurium was only recovered in seedpods exposed to HS soil. To explain this difference, the water-holding capacity (WHC) for each soil type was determined. At 25% WHC, HS soil required markedly less water to become saturated. PM with a WHC of 202% required a much greater volume of water to achieve saturation. This means that the movement of water within PM is restricted when IMC is low relative to the WHC. No *S.* Typhimurium was recovered from the interior of seedpods when pods were exposed to inoculated PM up to 40% IMC. The high WHC of PM is thought to be one factor influencing this result. When the WHC of a soil is extremely high, the amount of water required to allow free movement is greater than when WHC is low. Thus, in a water-restricted environment, *Salmonella* is unable to internalize within the seedpod.

WHC is just one way to characterize soil. In order to examine this relationship more fully, other methods for characterizing soil such as the porosity of the soil could be measured. It is important to note that initial and final concentrations of *S.* Typhimurium in soil was no different in both soil types at IMCs where internalization was observed in HS soil. This eliminates the growth of *Salmonella* in the matrix as a potential confounding variable. It is still plausible that when cells diffuse through the peanut pod

they are capable of replication under moist conditions. This may be especially important given the long exposure period tested. This idea could partially explain the high levels of *S.* Typhimurium detected on the interior surface when IMC was 30% in HS soil.

In an effort to examine the ability of *Salmonella* to migrate within fresh pods, experiments were conducted on GV peanut pods. While *S.* Typhimurium was recovered from the internal surface of the pod, the quantity recovered from the internal surface was significantly lower than what was observed in DV pods. It has been reported that the moisture content of walnut surfaces influences the survival of *Salmonella* (160), but the importance of pod moisture content on *Salmonella* internalization has yet to be examined. To understand differences in internalization between pod types, the moisture content of each pod type was measured. GV pods had moisture content of 55% and DV pods had moisture content of 4%. This difference may indicate that pod moisture content is a factor affecting the internalization of *S.* Typhimurium. To test this hypothesis, GV pods were also dried to below 10% moisture and internalization examined. The recovery of S. Typhimurium from dried GV (DGV) pods was 2.3 Log CFU/pod greater than GV pods when IMC for HS soil was 30%. While these results indicated that drying the pods allowed *S.* Typhimurium to internalize within the pod, the mechanism is still unknown. Water absorption of pods is greatly reduced in GV pods. Thus, it is likely that *S.* Typhimurium is simply carried with water through the pod explaining the reduction in internalization observed in GV pods. Internalized bacteria were recovered above the detection limit of the test before drying, at > 5.5 Log CFU/pod. Compared with DV pods in which native internalized bacteria were rarely observed on dTSA, the presence of these

microorganisms might influence the internalization of *Salmonella*. Thus, microbial competition for niche space cannot be ruled out as a factor influencing this result.

4.2 *Salmonella* persistence in soil

S. Typhimurium was only able to persist and grow in sterile HS soil for extended periods (Figure 5). After experiencing an initial lag phase, which varied between replicates, *S.* Typhimurium was able to consistently reach 6.4 Log CFU/g 11 days after inoculation. In all other soil matrices tested (sterile/non-sterile PM and non-sterile HS soil) the levels of S. Typhimurium declined quickly and were undetectable (< 2.0 Log CFU/g) after 6 days. Due to the high detection limit and the low inoculum level, the total reduction was only 2 Log CFU/g. Whether the population decreased more during this time is unknown. Islam et al. observed a similar 2 Log CFU/g reduction of *S.* Typhimurium in manure-amended soil at two weeks post inoculation (45). Therefore the result may not be atypical. The pH of the PM used was examined to potentially explain this result, but the observed pH of 6.6 will not significantly influence the population of *S.* Typhimurium. Competition for resources, availability of resources, the presence of toxic compounds have all been suggested to explain decreases in enteric bacterial populations in soil (162). Competition for resources may explain the decline in population observed in non-sterile HS soil, as *S.* Typhimurium was able to grow when the natural microflora were removed. In PM, there may be a toxic substance causing the reduction, as the reduction observed is under both sterile and non-sterile conditions.

It is important to note that initial and final concentration data collected for 20% IMC in PM during seedpod exposures differ from the results of time course experiments

(Figure 5). The concentration taken at the end of the exposure (3 days after inoculation) averaged 6.0 Log CFU/g. This is 0.1 Log CFU/g lower than the average initial concentration, which was 6.1 Log CFU/g. This discrepancy is not easily explained, but could be in part related to the presence of the peanut pod, which may provide additional niche space to allow the survival of *Salmonella*. This notion was not tested as the final concentration was determined upon mixing of the soil. More fidelity would have been provided if the initial inoculum level of *S.* Typhimurium in PM soil for the time course experiments was increased.

As was observed for the internalization of *S.* Typhimurium in intact seedpods through inoculated soil, the difference between the initial and final soil count of *S.* Typhimurium increased as the IMC of the soil decreased. The concentration decreased more than 1 Log CFU/g only at 4% IMC in HS soil. This is contrasted with PM in which the concentration decreased an average of 1.9 Log CFU/g when IMC was below 20%. This observation suggested that water was important for *Salmonella* to survive in the soil environment. It appeared that under dry conditions, the *S.* Typhimurium concentration did not decline. This could be studied over additional time points to understand if the population will plateau after an initial decline or if it will continue to decrease.

4.3 Internalization of *S.* Typhimurium in peanut plants

Salmonella was capable of remaining viable in sterile HS soil at high levels throughout the duration of the study (35 days), but the numbers declined by 1 Log CFU/g over this time period. This result is in agreement of Zheng et al. who studied the internalization of *S.* Newport in tomatoes through potting media (21). It has been

reported that *Salmonella* was capable of internalizing within the tissue of different plants, including peanuts (21, 50, 54, 56). This relationship was studied in peanuts by Deering et al. who germinated *S.* Typhimurium soaked seeds and visualized the localization of *Salmonella* within host tissue (56). In the present study, a longer incubation time was used to determine if *S.* Tyiphimurium was able to persist within peanut plants several weeks after infiltration. The overall results for this study were that *S.* Typhimurium was able to internalize within peanut plants and remain viable throughout the experiment (up to 35 days). This result indicated that *S.* Typhimurium could cope and adapt within plant tissue for extended periods. The mechanism by which *Salmonella* was able to survive and possibly grow (50, 54, 56) inside plant tissues is not fully understood, but it was suggested that *Salmonella* may reside in the apoplastic space of plant tissue and is capable of degrading cell wall components to use a nutrient source (57).

Recovery of *S.* Typhimurium was greater from stem samples than from root and leaf samples. The low concentration of cells recovered from leaf samples was expected and has been observed in other plants (21). Internalization in root tissue has been observed as well, but has been reported at greater levels than the stem and leaf tissue (55, 163). The sterilization procedure employed for this research may have influenced the *S.* Typhimurium count observed within root samples. Although the sterilization procedure was adjusted to fully sterilize entire plants, the absorption of bleach by roots during sterilization may have resulted in an underestimation in the *S.* Typhimurium count. While this observation was likely due to the harsh sterilization procedure, the phenomenon warrants further investigation.

This is the first to study on the internalization of *Salmonella* in peanut plants over time. It is interesting to note that the tissue localization pattern observed was different 28 days after sowing than14 days after sowing (Figure 11). Whether this indicates the relationship between bacterium and host changed over time is still yet unknown. The variation between tissue section increased at each testing point. As mentioned in Chapter 3, plants harvested for the third trial did not contain any internalized *S.* Typhimurium, which greatly increased the variation of the dataset. This observation was not made for the other two trials, but cannot be dismissed as an outlier in the dataset at this time. The detection limit was also relatively high at 50 CFU/g of sample. In the future, qualitative analyses such as traditional biochemical screening or PCR techniques can provide more insight into the ability of *Salmonella* to internalize peanut plants.

To date no other study has examined the possibility of *Salmonella* internalization in peanut plants through soil. This is the first report indicating that *Salmonella* was capable of internalizing within peanut plants through contaminated soil. We observed that sterile HS soil supported the growth of *S.* Typhimurium (Figure 3), making the active pursuit of a nutrient source within plant tissue less likely. A more probable explanation is that the mechanism is passive and that *S.* Typhimurium internalization occurred as water diffuses within host tissue as described by Deering et al. (57). This notion was established in part based on evidence provided by Solomon and Mathews who found that bacteria-sized polystyrene spheres absorbed through the roots of growing lettuce were found in leaf and stem tissue of the plant (164).

Nutrient sources may not be the only factor determining internalization. Environmental conditions, such as temperature, microbial competition, and water

availability all have the potential to influence internalization. Of particular importance in the current study was the availability of water throughout the growth of plants. Plants were irrigated every 2 days and were therefore subjected to periods of wet and dry conditions within soil. Indeed, the overall concentration of *S.* Typhimurium in soil during plant experiments fluctuated after inoculation and in one case (day 28) was as low as 4.5 Log CFU/g (Figure 9). Although the count in soil fluctuated, the concentration of *S.* Typhimurium in the rhizosphere may have fluctuated less.

Young reported that the rhizosphere can be wetter than bulk soil in which periods of drying occur (165). Thus, it follows suit that *S.* Typhimurium in the rhizosphere would be somewhat protected against fluctuations in water availability and maintain high levels of contamination. Whether *Salmonella* can actively pursue the rhizosphere through chemotaxis is still under debate (20, 52), but the environmental stability gained in the rhizosphere is still an important aspect to consider when assessing internalization. This notion only applies under sterile conditions, as microbial competition is likely to play a more significant role influencing *Salmonella* concentrations in a non-sterile environment. Unfortunately, the level of *S.* Typhimurium present in the rhizosphere was not measured but warrants further examination.

To assess the influence of, the nodulating symbiont, *Bradyrhizobium* on *Salmonella* internalization in peanut plants, peanut seeds were dip-inoculated and grown in *S.* Typhimurium inoculated HS soil. The average recovery was less in nodulated plants than plants with no nodules (Figure 13). However, no significant differences were observed between the recovery of *S.* Typhimurium from nodulated and non-nodulated plants. This implies that *Bradyrhizobium* NC92 did not impact the internalization of

Salmonella when seeds were germinated in inoculated soil. Because nodule formation increases the fitness of the plant, the importance of *Bradyrhizobium* on the internalization of *Salmonella* should be examined in more detail. Of particular importance is the examination of internalization when *Salmonella* is inoculated at difference stages of the plant life cycle. This is based on evidence that enteric pathogen internalization may be related to the time in the plants life cycle at which the pathogen is introduced to the environment (21). This is the first study to investigate the role of *Bradyrhizobium* on *Salmonella* internalization.

Overall, the results of the current study shed additional light on the relationship between the enteric pathogen and the plant host. In particular the results suggest that *Salmonella* is capable of internalizing within peanut tissue through sterile soil and that *Salmonella* predominates in the stems over the leaves and roots. If one is to examine peanut plants, the nodulating symbiont, *Bradyrhizobium* should be regarded as well. The findings of our study did not clearly support that *Bradyrhizobium* influenced the internalization of *Salmonella* in peanut plants germinated and grown in inoculated soil.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1. Conclusions

Several outbreaks involving peanut products and *Salmonella* occurring over the past 20 years prompted the investigation of factors that contribute to contamination of final product. The low water activity peanut butter was originally thought to be free of pathogen contamination, but recent outbreaks and current research has demonstrated that *Salmonella* can survive and maintain levels high enough to cause human illness (9). Although *Salmonella* has been examined in final products, there remains a need to investigate the relationship between the bacterium with raw peanut pods and plants. This is in effort to identify additional routes of entry for *Salmonella* in a peanut processing facility.

The current study evaluated the potential for *S.* Typhimurium to internalize peanut plants and seedpods. For suspension trials, *S.* Typhimurium was not only able to contaminate the interior surface of seedpods at a level of 6.1 Log CFU/pod when exposed for 22 h, but was also recovered from the interior surface of peanut pods after only 0.5 h of exposure (2.7 Log CFU/pod). In addition to this, overall results indicated that dried Virginia (DV) seedpods were more susceptible to *S.* Typhimurium internalization than undried or "green" Virginia (GV) seedpods when pods were exposed to contaminated soil. It was also observed that the soil initial moisture content (IMC) in relation to the soil water-holding capacity (WHC) impacted the ability of *S.* Typhimurium to internalize DV seedpods. This assertion is based on results in which *S.* Typhimuirum was recovered from a low WHC soil and was not recovered from a high WHC even at elevated levels of moisture. The results presented also suggest that the mechanism by which *S.*

Typhimurium internalized in seedpods was passive and that *S.* Typhimurium migrated with water absorbed by seedpods.

Additionally, the ability of *S.* Typhimurium to internalize in peanut plants was also examined. Overall findings are that *S.* Typhimurium can internalize in peanut plant tissue when seeds are germinated in *S.* Typhimurium a contaminated soil. Stems were found to contain the most internalized *S.* Typhimurium at 3.5 Log CFU/g. Furthermore, no significant differences in internalization were observed when plants were inoculated with and without the peanut symbiont, *Bradyrhizobium*.

The results presented represent the first investigation at the ability of *Salmonella* to internalize peanut seedpods. Given that contamination was largely dependent on available moisture, the importance of maintaining a dry facility should be stressed. This may prove difficult as *Salmonella* was able to contaminate the interior surface of seedpods in Hubbard series soil with an initial moisture content of 15%. The supply chain and impact of transportation should also be examined to identify potential contamination sites. The US Food and Drug Administration published a report on the environmental and product testing results of the Sunland Inc. facility in which several products and surfaces were positive for *Salmonella* (151). With this as an example, it is important to note that once *Salmonella* gains entry into a facility, the spread of the pathogen can lead to contamination of final product, ending in human illness.

5.2. Future work

The work presented identifies that *S.* Typhimurium has the potential to contaminate the interior of peanut plants and seedpods. Future work should examine this

relationship using different serovars and peanut varieties. Although *S.* Typhimurium was chosen for study in this experiment, other outbreak strains should be examined such as *S.* Tennessee, *S.* Mbandaka, *S.* Newport, and *S.* Bredeney. The study examined internalization, but did not investigate the ability of *Salmonella* to be absorbed through the root system of plants at different times in the plant's life cycle or the ability of the plant to attach and interact with other plant tissues. The importance of microbial diversity in a field cannot be overlooked and should be examined as well. The impact that non-sterile soil has on internalization could also be another interesting area of study as it has been demonstrated by these findings that the concentration of *Salmonella* in a competitive environment does not persist over time. Additionally, fumigants used on farms have been known to alter soil ecology possibly creating a niche for *Salmonella* to survive and impact the safety of peanut crops.

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APPENDIX

6.1. Experimental data

Table A.1: Experimental data for suspension trials. Individual replicate data points for Figures 1, 2, and 3. ND = "not detected"

Exposure time (h)	Drying time (h)	Temp (°C)	Suspension (Log CFU/mL)	Recovery (Log CFU)		Pod mass (g)		
				Seed	Internal Surface	Before	After	Diff.
2	24	25	8.94	ND	ND	ND	ND	ND
2	24	25	8.94	ND	2.81	ND	ND	ND
2	24	25	8.94	ND	3.35	ND	ND	ND
4	24	25	8.04	2.00	ND	ND	ND	ND
4	24	25	8.04	2.48	4.61	ND	ND	ND
4	24	25	8.04	ND	2.00	ND	ND	ND
4	24	25	8.04	>5.48	5.78	ND	ND	ND
4	24	25	8.04	2.90	3.94	ND	ND	ND
24	1	25	7.71	ND	2.30	ND	ND	ND
24	1	25	7.71	3.00	5.15	ND	ND	ND
24	1	25	7.71	ND	2.04	ND	ND	ND
24	1	25	7.71	2.85	4.38	ND	ND	ND
24	1	25	7.71	ND	4.05	ND	ND	ND
0.55	0	25	7.83	ND	ND	ND	ND	ND
0.55	0	25	7.83	ND	ND	ND	ND	ND
0.55	0	25	7.83	ND	2.63	ND	ND	ND
1	0	25	7.45	ND	3.96	ND	ND	ND
1	0	25	7.45	5.66	5.62	ND	ND	ND
1	0	25	7.45	ND	2.29	ND	ND	ND
1	0	25	7.45	3.72	5.36	ND	ND	ND
1	0	25	7.45	2.00	5.59	ND	ND	ND
0.5	0	25	7.90	ND	4.79	3.25	3.38	0.13
0.5	0	25	7.90	ND	2.11	2.82	3.02	0.20
0.5	0	25	7.90	ND	3.87	3.28	3.55	0.27
1	0	25	7.90	ND	ND	2.86	3.12	0.26
1	0	25	7.90	ND	4.08	2.83	3.07	0.24

Exposure time (h)	Drying time (h)	Temp (°C)	Suspension (Log CFU/mL)	Recovery (Log CFU)		Pod mass (g)		
				Seed	Internal Surface	Before	After	Diff.
1	0	25	7.90	ND	ND	3.83	4.09	0.26
1.5	0	25	7.90	3.99	5.20	2.90	3.36	0.46
1.5	0	25	7.90	ND	3.73	2.98	3.26	0.28
3	0	25	7.64	ND	2.30	3.65	4.09	0.44
3	0	25	7.64	2.36	4.35	3.15	3.67	0.52
3	0	25	7.64	ND	4.80	2.58	2.96	0.38
1	0	25	7.41	1.83	2.92	3.04	3.26	0.22
1	0	25	7.41	1.52	3.00	3.40	3.76	0.36
1	0	25	7.41	ND	2.64	3.46	3.70	0.24
1	0	25	7.41	ND	2.30	2.99	3.14	0.15
24	0	25	7.41	2.95	4.38	3.26	4.35	1.09
24	0	25	7.41	4.23	5.76	2.76	3.63	0.87
24	0	25	7.41	3.52	4.70	3.00	3.69	0.69
24	0	25	7.41	3.96	4.08	3.02	3.91	0.89
0.5	0	37	7.45	ND	ND	3.33	3.66	0.33
0.5	0	37	7.45	2.36	4.35	2.32	2.56	0.24
1.5	0	37	7.45	3.69	3.47	2.81	3.14	0.33
1.5	0	37	7.45	3.20	3.95	3.17	3.67	0.50
1.5	0	37	7.45	4.64	5.30	3.66	4.15	0.49
0.5	0	4	7.45	2.11	4.10	3.13	3.41	0.28
0.5	0	4	7.45	2.30	3.77	2.26	2.39	0.13
1.5	0	4	7.45	3.78	4.03	3.23	3.62	0.39
1.5	0	4	7.45	ND	4.35	2.68	3.16	0.48
2	2	25	7.40	ND	2.11	3.28	3.43	0.15
2	2	25	7.40	2.52	3.36	3.39	3.50	0.11
2	2	25	7.40	ND	3.28	3.11	3.52	0.41
2	4	25	7.40	ND	ND	2.54	2.68	0.14
2	4	25	7.40	ND	ND	2.11	2.46	0.35
2	4	25	7.40	ND	2.36	3.17	3.46	0.29
2	6	25	7.40	ND	ND	3.52	3.79	0.27
2	6	25	7.40	ND	ND	3.21	3.61	0.40
2	6	25	7.40	ND	3.76	3.19	3.44	0.25
2	0	25	2.00	ND	ND	3.09	3.65	0.56
2	0	25	2.00	ND	ND	2.9	3.62	0.72
2	0	25	2.00	ND	ND	2.42	2.8	0.38
2	0	25	3.96	ND	ND	3.32	3.96	0.64

Exposure time (h)	Drying time (h)	Temp (°C)	Suspension (Log CFU/mL)	Recovery (Log CFU)		Pod mass (g)		
				Seed	Internal Surface	Before	After	Diff.
2	0	25	3.96	3.54	2.70	2.8	3.5	0.70
2	0	25	3.96	2.52	3.21	3.15	3.86	0.71
2	0	25	6.07	ND	ND	1.93	2.21	0.28
2	0	25	6.07	ND	ND	3.21	3.62	0.41
2	0	25	6.07	ND	ND	3.2	3.67	0.47
16	0	25	7.93	4.00	4.48	3.23	4.20	0.97
16	0	25	7.81	1.36	2.47	2.43	3.07	0.64
16	0	25	7.89	5.47	5.39	2.47	3.15	0.68
16	0	4	7.78	2.38	3.75	2.72	3.37	0.65
16	0	4	7.84	1.95	3.00	2.88	3.51	0.63
12	0	4	7.34	2.82	3.05	2.90	3.57	0.67
12	0	4	7.34	1.96	3.12	2.45	2.94	0.49
12	0	4	7.34	2.29	2.85	1.97	2.51	0.54
12	0	25	7.34	>5.62	4.92	2.82	3.50	0.68
12	0	25	7.34	5.17	5.30	3.19	3.97	0.78
12	0	25	7.34	1.74	3.63	3.10	4.04	0.94
4	0	4	7.24	2.64	3.02	2.66	3.09	0.43
4	0	4	7.24	3.12	3.72	3.00	3.43	0.43
4	0	25	7.24	1.88	4.42	2.84	3.49	0.65
4	0	25	7.24	3.19	3.86	3.80	4.03	0.23
4	0	37	7.24	4.81	4.99	2.91	3.53	0.62
22	0	4	7.24	4.89	4.95	2.96	3.68	0.72
22	0	4	7.24	3.56	4.43	2.71	3.28	0.57
22	0	25	7.24	6.16	5.55	3.59	4.81	1.22
22	0	25	7.24	5.83	5.26	3.3	4.3	1.00
22	0	37	7.24	6.27	6.08	2.49	3.19	0.70
2	0	25	5.40	ND	2.80	2.97	3.32	0.35
2	0	25	5.40	ND	1.70	2.99	3.4	0.41
2	0	25	3.44	ND	ND	2.67	3.2	0.53
2	0	25	3.44	ND	1.48	2.85	3.36	0.51
2	0	25	2.35	ND	ND	3.23	3.92	0.69
2	0	25	2.35	ND	ND	2.65	2.96	0.31
22	0	16	7.32	2.53	4.53	3.09	3.95	0.86
22	0	16	7.32	0.90	3.54	2.86	3.78	0.92
22	0	16	7.32	ND	2.20	2.84	3.52	0.68
22	0	42	7.32	3.73	ND	2.56	3.51	0.95

Exposure time (h)	Drying time (h)	Temp (°C)	Suspension (Log CFU/mL)	Recovery (Log CFU)		Pod mass (g)		
				Seed	Internal Surface	Before	After	Diff.
22	0	42	7.32	3.86	4.52	2.65	3.20	0.55
22	0	42	7.32	>5.62	ND	3.11	3.94	0.83
22	0	42	7.43	5.01	6.41	2.73	3.54	0.81
22	0	42	7.43	3.86	5.89	2.63	3.56	0.93
22	0	42	7.43	4.44	5.55	3.41	4.48	1.07
22	0	37	7.40	4.92	6.29	3.25	4.42	1.17
22	0	37	7.40	4.84	6.34	3.40	4.25	0.85
22	0	37	7.40	5.74	6.77	2.76	3.6	0.84
4	0	16	7.64	0.48	3.56	2.98	3.37	0.39
4	0	16	7.64	1.30	3.70	3.21	3.78	0.57
4	0	16	7.64	3.36	4.47	2.94	3.5	0.56
4	0	42	7.64	0.70	5.09	2.53	3.08	0.55
4	0	42	7.64	4.63	3.56	2.74	3.34	0.6
4	0	42	7.64	ND	3.14	2.39	3.83	1.44
4	0	37	7.62	3.69	4.71	3.47	4.46	0.99
4	0	37	7.62	ND	5.84	2.79	3.22	0.43

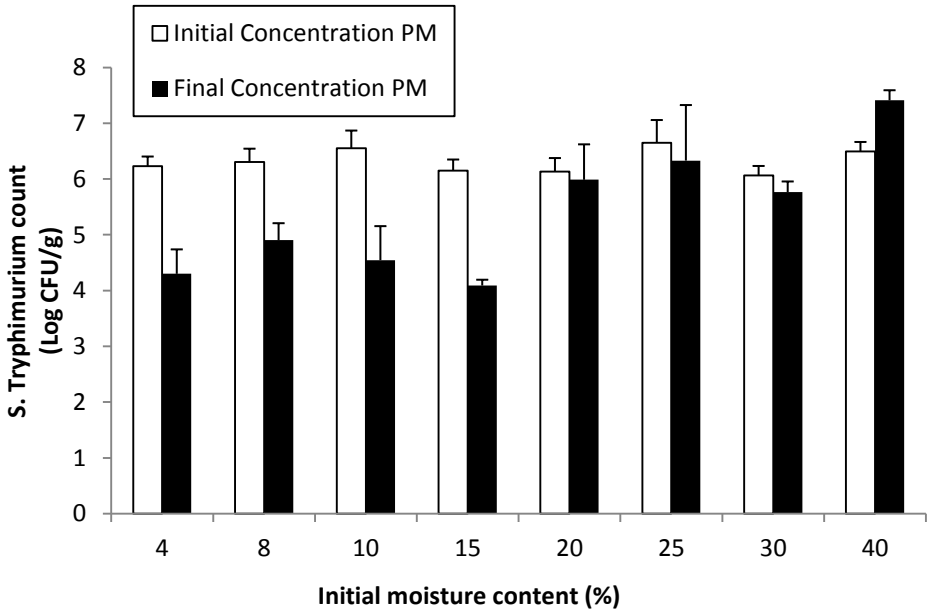

Figure A.1: Initial and final count of S. Typhimurium in PM for DV seedpod exposure experiments

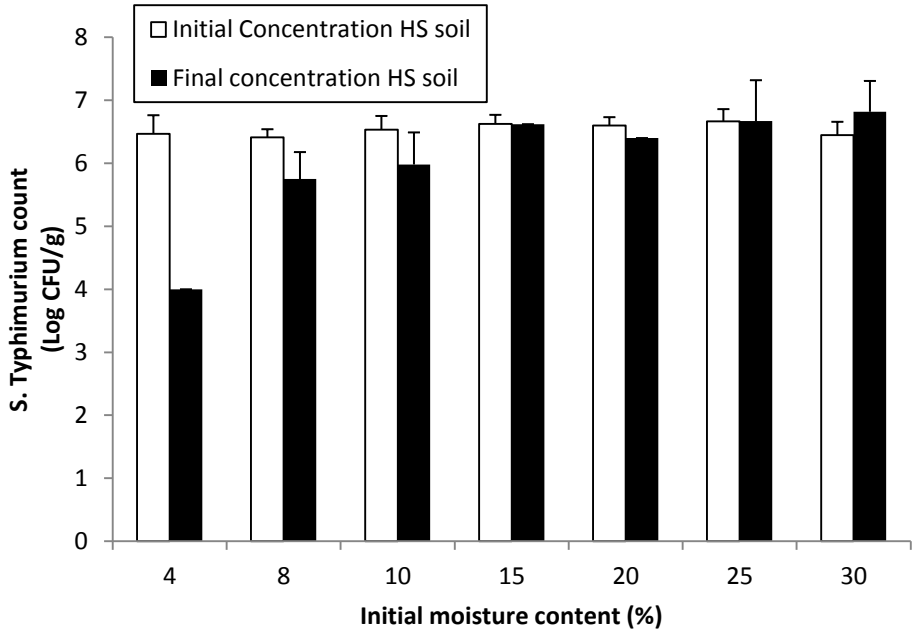

Figure A.2: Initial and final count of S. Typhimurium in HS soil for DV seedpod exposure experiments

Table A.2: Experimental data for soil exposure trials. Individual data points for Figure 6, and Table 6. ND = "not detected"

Initial moisture content (%)	Soil count (Log CFU/g)		Recovery (Log CFU/pod)	
	Initial count	Final Count	Seed	Internal Surface
Hubbard series soil				
4	6.15	4.00	ND	ND
4	6.50	ND	ND	ND
4	6.70	ND	ND	ND
8	6.54	5.41	ND	ND
8	6.28	5.61	ND	ND
8	6.41	6.23	ND	ND
10	6.71	5.62	ND	ND
10	6.58	6.34	ND	ND
10	6.28	ND	ND	ND
15	6.50	ND	ND	ND
15	6.80	ND	1.48	2.58
15	6.70	ND	ND	1.53
15	6.52	6.62	1.98	4.20
20	6.48	6.40	1.50	3.17
20	6.92	5.73	1.96	2.68
20	6.70	ND	2.24	3.08
25	6.66	6.77	1.40	2.15
25	6.63	7.18	3.04	4.91
25	6.45	ND	3.32	5.27
30	6.80	ND	1.30	3.99
30	6.49	7.30	3.32	5.17
30	6.52	6.83	2.96	4.80
30	6.32	6.32	2.20	4.41
Potting media				
4	6.11	ND	ND	ND
4	6.15	4.61	ND	ND
4	6.43	3.99	ND	ND
8	6.30	5.04	ND	ND
8	6.59	4.96	ND	ND

Initial moisture content (%)	Soil count (Log CFU/g)		Recovery (Log CFU/pod)	
	Initial count	Final Count	Seed	Internal Surface
8	6.00	5.15	ND	ND
8	6.32	4.46	ND	ND
10	6.20	4.70	ND	ND
10	6.26	4.18	ND	ND
10	6.58	3.70	ND	ND
10	6.89	5.26	ND	ND
10	6.83	4.88	ND	ND
15	5.96	4.18	ND	ND
15	6.36	4.00	ND	ND
15	6.28	4.18	ND	ND
15	6.00	4.00	ND	ND
20	6.41	5.97	ND	ND
20	5.95	6.63	ND	ND
20	6.03	5.36	ND	ND
25	6.91	7.48	ND	ND
25	6.86	5.79	ND	ND
25	6.18	5.72	ND	ND
30	6.28	ND	ND	ND
30	5.95	5.63	ND	ND
30	6.18	5.90	ND	ND
40	6.69	7.20	ND	ND
40	6.40	7.52	ND	ND
40	6.40	7.51	ND	ND

Table A.3: Experimental data for green pods and dry green pods for soil exposure experiments. Replicate data for Figure 7. ND = "not detected"

Initial moisture content (%)	Soil count (Log CFU/g)		Recovery (Log CFU/pod)	
	Initial count	Final Count	Seed	Internal Surface
Green pods				
20	6.24	6.00	ND	ND
20	6.37	6.00	1.23	1.23
20	6.00	5.66	ND	ND
30	6.23	5.41	ND	ND
30	6.34	5.20	2.20	1.26
30	6.32	5.38	1.52	1.81
Green dry pods				
20	6.24	5.48	1.65	3.01
20	6.00	5.48	ND	1.28
20	6.41	6.15	2.35	2.51
30	6.32	5.08	2.4	3.81
30	6.23	6.26	3.19	3.82
30	6.18	5.90	2.45	3.18

Table A.4: Gravimetric determination of soil water-holding capacity (WHC). Replicate data.

Soil type	WHC (%)	Average	Standard deviation
Hubbard series	29		
	23		
	22	24.70%	3.92%
Potting media	201		
	213		
	192	202.02%	10.24%

Table A.5: *S.* Typhimurium counts recovered for plant internalization experiments. Individual data points used for Figure 12. ND = "not detected"

Days post planting	Internalized *S.* Typhimurium (Log CFU/g)
Roots	
14	2.83
14	2.10
14	1.87
21	3.20
21	1.97
21	3.35
28	4.01
28	2.01
28	ND
Stems	
14	3.37
14	4.16
14	3.38
21	3.49
21	3.07
21	4.16
28	4.10
28	3.64
28	ND
Leaves	
14	1.79
14	ND
14	ND
21	1.70
21	ND
21	1.79
28	1.70
28	ND
28	ND

Table A.6: Internalization of *S.* Typhimurium with and without *B.* NC92 inoculation. Individual replicate for Figure 12. ND = "not detected"

Days post planting	Internalized *S.* Typhimurium (Log CFU/g)	
	with *B.* NC92	Control
Roots		
21	ND	3.50
21	ND	ND
28	2.40	4.62
28	4.27	4.04
35	3.50	3.97
35	ND	ND
Stems		
21	2.11	4.16
21	4.15	4.45
28	3.24	4.57
28	4.15	6.50
35	4.02	4.32
35	4.23	4.71
Leaves		
21	ND	ND
21	ND	3.12
28	ND	2.80
28	ND	ND
35	ND	ND
35	ND	ND

Table A.7: Moisture content of soil during watering cycle of growing plants. Replicate data.

Time ofter watering (h)	Moisture Content	Average	Standard Deviation
		(%)	
16	19.35		
16	19.65		
16	20.92	19.98	0.83
48	8.02		
48	9.72		
48	8.33	8.69	0.91